Universidade de Lisboa

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LECTINS IN MEDITERRANEAN FLORA: PROTEOMICS AND ANTITUMORAL ACTIVITY IN COLON CANCER CELLS

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Aos meus avós

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Abstract

Lectins are proteins capable of specifically binding to glycans. This characteristic makes them unique molecules in cell recognition, especially of anomalous cells, since they have on their surface characteristic receptors. In addition to being of extreme importance in cell recognition, lectins have been described as capable of inducing cell death in various types of cancer, through several processes, namely, apoptosis and autophagy. Thus, because lectins are widely distributed in nature, with plants being one of the largest reservoirs, and have particular characteristics, these proteins are a topic of intense research.

Colorectal cancer is the third most common cancer in men and the second most common in women. Phenotypic changes may be due to post-translational modifications, namely, changes in protein glycosylation, more specifically in *N*-glycosylation. *N*-glycans are of extreme importance in tumorigenesis, since they are involved in several cellular mechanisms, such as metabolization, signaling, growth, cell adhesion, cell-matrix interaction, invasion and metastasis.

The studies presented in this thesis aimed to discovery new lectins, present in Mediterranean flora species, with capacity to specifically interact with colorectal cancer cells and induce cell death.

After protein separation of lectins present in the protein extracts of the selected species, *Arbutus unedo*, it was found that the protein of interest appears to have a molecular weight of ≈ 15 kDa, being able to interact with HT29 human colon cancer cell membranes. In antitumor activity assays in HT29 cells, the purified protein increased cell death and decreased cell viability. However, when compared to the protein extract, the purified protein showed less activity in both assays. Caspases-3/7 activity assays and nuclear fragmentation assays suggest that cell death may not occur through apoptosis under these experimental conditions.

In conclusion, we show the presence of lectins in the studied species, and the purified lectin demonstrated binding capacity and antitumor activity for the HT29 cells.

Key Words: Lectins; colorectal cancer; aberrant glycosylation; apoptosis; necrosis

Resumo

As lectinas são proteínas com capacidade de se ligarem de forma específica a glicanos. Esta característica torna-as moléculas únicas no reconhecimento de células, nomeadamente de células anómalas, uma vez que estas têm na sua superfície recetores glicosilados característicos. Para além de serem de extrema importância no reconhecimento celular, as lectinas têm sido descritas como tendo capacidade de induzir morte celular em diversos tipos de cancro, através de inúmeros processos, nomeadamente apoptose e autofagia. Assim, pelo facto de se encontrarem extensamente distribuídas na natureza, sendo as plantas um dos maiores reservatórios de lectinas, e devido às suas características, estas proteínas são alvo de intensa investigação.

O cancro colon retal é o terceiro cancro mais comum nos homens e o segundo mais comum nas mulheres. O desenvolvimento e progressão deste tipo de cancro deve-se à acumulação de alterações genéticas, nomeadamente alterações que afetam a proliferação, a sobrevivência, a migração e a invasão destas células para outros tecidos. Ocorrem também alterações fenotípicas, devido a modificações pós-tradução, incluindo modificações na glicosilação proteica, mais em concreto da *N*-glicosilação. Os *N*-glicanos são de extrema importância no processo tumoral, uma vez que estão envolvidos em diversos mecanismos celulares, como a metabolização, a sinalização, o crescimento, a adesão entre células, a interação célula-matriz, a invasão e a metastização. Todas estas alterações conduzem ao avanço progressivo da doença sendo que, apesar dos significativos avanços no tratamento do cancro colon retal, os doentes metastáticos apesentam uma taxa de sobrevida a 5 anos limitada a 13%. É, por isso, imperativo encontrar novas alternativas terapêuticas.

Os estudos apresentados nesta tese tiverem como principal objetivo a procura de novas lectinas, presentes em espécies da flora mediterrânea, com capacidade de interagirem de forma específica com as células do cancro colon retal e induzirem morte celular. Selecionamos quatro espécies da flora mediterrânica, sendo algumas endémicas da flora portuguesa (*Juniperus oxycedrus* subsp. *oxycedrus*, *Juniperus oxycedrus* subsp. *badia*, *Arbutus unedo* e *Corema album*). Todas exibem atividade hemaglutinante ao nível do extrato proteico, sugerindo a presença de proteínas do tipo lectinas. Estas espécies foram avaliadas em termos de atividade antitumoral, sendo que prosseguimos com a espécie que apresentou melhores resultados (*Arbutus unedo*), de forma a isolarmos a(s) lectina(s) responsável(eis). Para a lectina purificada fez-se a sua caracterização

proteómica e avaliou-se a sua atividade antitumoral assim como os mecanismos conducentes à sua citotoxicidade sobre as células humanas HT29, do cancro do cólon.

Os resultados apresentados mostram que todas as espécies estudadas têm capacidade de induzir morte celular e diminuir o metabolismo celular. No entanto, o *Arbutus unedo* foi a espécie que mais se destacou, quando comparado com os resultados anteriores obtidos nestas espécies. Para a espécie seleccionada, *Arbutus unedo*, após separação proteica das lectinas presentes no extrato, verificou-se que a proteína de interesse aparenta ter um peso molecular de ≈ 15 kDa, sendo capaz de se ligar às membranas das células HT29. Em relação aos ensaios de atividade antitumoral nas células HT29, a lectina purificada apresenta capacidade de aumentar a morte celular e diminuir a viabilidade celular; no entanto, quando comparado com o extrato proteico, este apresenta melhores resultados em ambos os ensaios. No que diz respeito ao mecanismo de morte associado a estas proteínas, após avaliar a atividade das caspases-3/7 e a presença de corpos apoptóticos, chegámos à conclusão que a apoptose poderá não ser a forma de morte celular predominante nestas condições experimentais.

Em suma, os resultados obtidos são bons indicadores da presença de lectinas, na espécie estudada, sendo que a lectina purificada demonstrou capacidade de ligação e atividade antitumoral para as células HT29, não se confirmando, no entanto, um mecanismo por apoptose. Este resultado final não invalida que se prossiga com a purificação de outras lectinas, também presentes no extrato proteico desta espécie e posterior avaliação do seu efeito a nível das células tumorais, uma vez que outras proteínas, com diferentes pesos moleculares, nomeadamente ≈ 29 kDa e ≈ 50 kDa, presentes no extrato proteico do *Arbutus unedo*, se ligaram igualmente às membranas das células HT29. As diferentes frações proteicas obtidas durante a purificação de lectinas revelaram U.H. (unidade hemaglutinante) diferentes, tendo a fração não adsorvida à coluna Q-Sepharose uma U.H. menor que a fração não adsorvida à coluna Mono S. O extrato total apresentou melhores resultados ao nível da atividade antitumoral do que a lectina purificada.

Palavras Chaves: Lectinas; cancro colon retal; glicosilação aberrante; apoptose; necrose

Abbreviations

Aun	*Arbutus unedo*
BAX	Gene coding for the pro-apoptotic protein bax
bax	Bcl-2 associated X protein
BCL-2	Gene coding for the anti-apoptotic protein bcl-2
Bcl-2	b-cell lymohoma protein 2
BSA	Bovine Serum Albumin
ConA	concanavalin A
Cal	*Corema album*
CRC	colorectal cancer
CRD	Carbohydrate-Recognition Domain
DTT	Dithiothreitol
EDTA	ethyenediaminetetraacetic acid
H.U.	Hemagglutination unit
Joba	*Juniperus oxycedrus* subsp. *badia*
Joox	*Juniperus oxycedrus* subsp. *oxycedrus*
LDH	lactate dehydrogenase
MMP	matrix metalloproteinase
MLs	mistletoe lectin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
SEM	standart error of mean
SDS-PAGE	Sodium dodecyl sulfate - Polyacrylamide gel electrophoresis
Tris-HCl	Tris (hydroxymethyl) aminomethane Hydrochloride

Chapter I –Introduction

1. Lectins

1.1. Definition

Lectins are a group of highly diverse proteins (Silva *et al.*, 2014), with non-immune origin, which bind in a reversible and specific manner to glycan and other molecules containing glycosylated receptors through hydrogen bonds and Van der Waals interactions (Lis and Sharon, 1998; Vasconcelos and Oliveira, 2004). These proteins may be used to precipitate glycoconjugates (Goldstein *et al.*, 1980; Van Damme *et al.*, 1998). Furthermore, lectins have at least one non-catalytic domain that enables them to selectively recognize mono-, poly- or oligosaccharides, and to bind without altering the structure of the glycan (Silva *et al.*, 2013; Silva *et al.*, 2014).

The first lectin described was in 1888 by Hermann Stillmark, who observed the agglutination of erythrocytes, while studying the toxic effect of castor bean seed extracts (*Ricinus communis* L.) in blood (Stillmark, 1888). Since then, the word agglutinin has been extensively used to describe molecules with capacity to agglutinate erythrocytes and other cells (Hartley and Lord, 2004). Only in 1954 the term lectin (from the Latin verb *legere*, which means "to select") was proposed by William Boyd (Boyd and Shapleigh, 1954; Lam and Ng, 2011) to referred to the ability of some carbohydrate-binding proteins to selectively agglutinate erythrocytes of a particular human blood group (Peumans and Damme, 1995).

1.2. Occurrence of lectins

Lectins are widely distributed in nature and can be found in almost all cells and secretome membranes of all living organisms, including plants, algae, fungi, animals (vertebrates and invertebrates), microorganisms and viruses (De Hoff *et al.*, 2009; Faheina-Martins *et al.*, 2012).

In all tissues of plants, like leaves, stems, bark, bulbs, tubers, corms, rhizomes, phloem, fruits, roots and flower tissues we can found lectins, and due their specific carbohydrate binding they are able to recognize hormones, nucleic acids and non-protein amino acids (De Mejia and Prisecaru, 2005). This characteristic and its great distribution in nature shows that lectins are very important selective proteins.

1.3. Biological role of plant lectins and their application

Currently, lectins have become the focus of intense interest for biologists, in particular for the research and begins to be use in agriculture and medicine (Movafagh *et al.*, 2013) due to their highly specific interaction with glycans and the associated biological effects. The idea that lectins and glycans are excellent as cell recognition and interaction markers, cell adhesion, migration and invasion, originates from the findings that both classes of compound are commonly present on the cell surface (Figure I.1) and that glycans possess tremendous coding capacity (Ambrosi *et al.*, 2005; Ribeiro *et al.*, 2012).

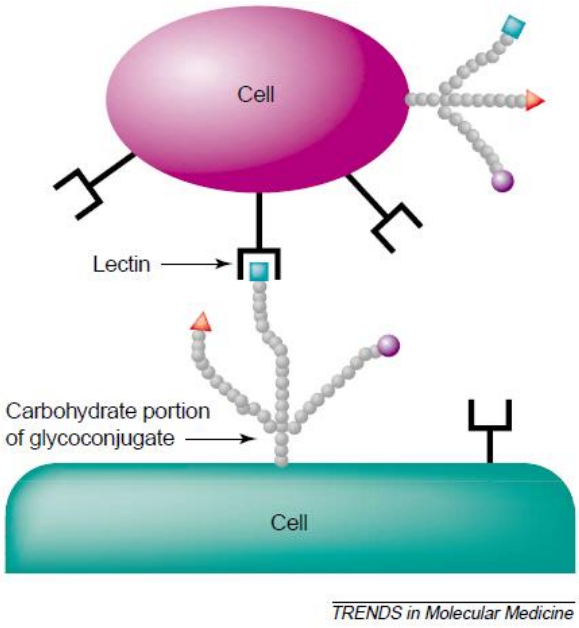


Figure I.1 – Lectin binding to glycoconjugate promoting cell-cell interaction.
Adapted from Nangia-Makker *et al.*, 2002

In plant, lectins play vital self-defending roles by deleteriously affecting the growth of insects or hindering oviposition behavior (Sadeghi *et al.*, 2006). The vulnerable plant organs like seeds usually possess an abundance of lectins (Worbs *et al.*, 2011). Expression of lectins in plants is sensitive to stress factors and environmental variations, like insect herbivory, drought, wounding, salt stress, hormone treatment and pathogen invasion (Zhang *et al.*, 2000; Lannoo and Van Damme,

2010). Tobacco leaves normally express a very low level of lectin, however, when treated with jasmonates or threatened by insect herbivory, lectin expression was found to be upregulated (Chen *et al.*, 2002; Lannoo and Van Damme, 2010). Plant lectins may also be involved in the interactions between plant and soil bacteria to establish a symbiotic relationship. For example, legume lectins contribute to the binding of rhizobia to the roots and formation of nodules (De Hoff *et al.*, 2009), that have a better nitrogen fixation capacity.

The plant lectins, in human cells, are well known to possess anti-tumor activity via programmed cell death (PCD), allowing to keep homeostasis. Several plant lectins such as mistletoe lectin (MLs), and ricin (RCA) have antiproliferative activity and induce apoptosis in cancer cells. However, other lectins such as Concanavalin A (ConA) and *Polygonatum cyrtonema* lectin (PCL) may result in cell death after internalization by autophagy or binding receptors that contain certain glycans on the surface of cancer cells (Fu *et al.*, 2011).

1.4. Classification

Lectins are a highly diverse group of proteins that include several families of proteins that generally, differ for biochemical/physicochemical properties, molecular structure, the binding specificity to carbohydrates and biological activities (Loris, 2002; Gallegos *et al.*, 2014). The classification of lectins in families is a challenge, however, of great importance, since it allows a better understanding of their functions. Thus, lectins, in general, can be classified based on binding specificity to the carbohydrate, in view of the monosaccharide to which exhibit higher affinity and considering the general structure; according to the recognition domains (RCD) for glycans and on the structure and evolutionary relationship.

1.4.1. Classification based on carbohydrate specificity

Lectins were initially classified, from a practical point of view, based on their specificity for different blood groups, taking into account the phenomenon of hemagglutination and subsequently the carbohydrate(s) identity that specifically binds to lectins provoking the inhibition of the hemagglutination (Rego *et al.*, 2002).

According to the monosaccharide ligand toward which lectins exhibit the highest affinity, Sharon (1998) classified lectins into several groups:

- Mannose/Glucose (group I)
- Galactose/*N*-Acetylgalactosamine (group II)
- *N*-Acetylglucosamine (group III)
- *L*-Fucose (group IV)
- Sialic acid (group V)
- Specific lectin to complex oligosaccharides and polysaccharides

However, this classification ignores mannose-6-phosphate and *N*-acetylgalactose-4-sulfate (Lis and Sharon, 1998; Ambrosi *et al.*, 2005).

1.4.2. Classification based on the structure

As more lectins with diverse sugar-binding specificities were gradually identified and taking into account that different carbohydrate-binding motifs can recognize similar sugar structures, the old system was no longer feasible, and another classification system were proposed.

Based on the overall domain structure of mature plant lectins, Van Damme and colleagues (1998), introduced another classification system in which they divided into four main classes: merolectins, hololectins, chimerolectins and superlectins (Van Damme *et al.*, 1998).

The **merolectins** are proteins that have only one binding domain glycans (Vasconcelos and Oliveira, 2004) consist of small individual polypeptides, because of their monovalent nature, are unable to agglutinate cells or precipitate glycoconjugates (Peumans and Damme, 1995).

The **hololectins** comprise all lectins that have the glycan binding domains (Vasconcelos and Oliveira, 2004), are able to agglutinate cells and precipitate glycoconjugates. Obviously, most of all plant lectins subtypes are hololectinas, since they behave as hemagglutinin (Peumans and Damme, 1995).

The **chimerolectins** are proteins consisting of one or more binding domains glycans with the addition of catalytic and biological activity, dependent on the different domain than the glycan binding (Vasconcelos and Oliveira, 2004).

The **superlectins** at least have two glycan binding domains but differ of hololectinas because of their binding sites are capable of recognizing not structurally related sugar (Vasconcelos and Oliveira, 2004).

1.4.3. Classification based on the structure and evolutionary relationships

More recently Van Damme and colleagues (2008) developed a new plant lectins classification system taking into account all the data that have become available in recent decades. According to the analysis of the genome/transcriptome plant lectins can be classified into 12 distinct families (**Table 1**) with domains evolutionarily and structurally related, such as (1) *Agaricus bisporus* agglutinin homologs, (2) Amaranthins, (3) Class V chitinase homologs with lectin activity, (4) Cyanovirin family, (5) EEA family, (6) GNA family, (7) Proteins with hevein domains, (8) Jacalins, (9) Proteins with legume lectin domains, (10) LysM domain, (11) Nictaba family (formerly *Cucurbitaceae phloem* lectins), (12) Ricin-B family (Van Damme *et al.*, 2008; De Hoff *et al.*, 2009; Fu *et al.*, 2011).

Table I-1 – The classification of plant lectins. Relation between structural and evolutionary characteristics and sugar-biding specificity classification.

Representative lectin	Abbreviation	Family	Sugar-biding specificity
***Agaricus bisporus* agglutinin**	ABA	*Agaricus bisporus* agglutinin homologs	Galactose
		Amaranthins	
Chitinase-related agglutinin	CRA	Class V chitinase homologs with lectin activity	High-mannose *N*-glycans
Cyanovirin-N	CV-N	Cyanovirin family	Mannose
***Euonymus europaeus* agglutinin**	EEA	EEA family	Mannose/galactose
***Polygonaturn cyrtonema* lectin**	PCL	GNA family	Mannose/sialic acid
Wheat germ agglutinin	WGA	Proteins with hevein domains	*N*-acetyl-D-glucosamine
Jacalin	JAC	Jacalins	Mannose
Concanavalin A	ConA	Proteins with legume lectin domains	D-mannose
		LysM domain	
***Cucurbitaceae phloem* lectin**	CPL	Nictaba family	
European mistletoe lectin	ML-I	Ricin-B family	*B*eta-galactose

Adapted from Fu *et al.*, 2011

Recent studies show that previously reported carbohydrate-binding affinities are sometimes misleading for lectin classification, because lectins, as determined by glycoarray analyses,

generally show higher binding affinities to complex oligosaccharides than to simple oligosaccharides or monomers (Van Damme *et al.*, 2008). For this reason, the traditional methods of carbohydrate monomer-based lectin classification are being supplanted by the more informative homology-based systems (De Hoff *et al.*, 2009). Each lectin domain has its own characteristic overall fold with one or more carbohydrate-binding sites (Vandenborre *et al.*, 2011). The occurrence of a particular carbohydrate-binding domain is in most cases not restricted to a certain plant family. Moreover, most of these domains are spread all over the plant kingdom (Jiang *et al.*, 2010).

1.5. Molecular structure

The structural studies of lectins, made by crystal structure, have shown that the different lectin families are generally structurally unrelated. Due to this fact, their recognition sites are highly variable, causing the different lectin families to exhibit different carbohydrate binding specificities (Loris, 2002).

Lectins of leguminous plants are the group of plant lectins best studied and characterized. Lectin Concanavalin A (Con A, **Figure I.2 and Figure I.3**) belongs to this group and was the first lectin to have resolved its three-dimensional structure, so far, the lectin best characterized. Therefore, in this work in relation to the molecular structure Con A and this lectin family will receive greater emphasis.

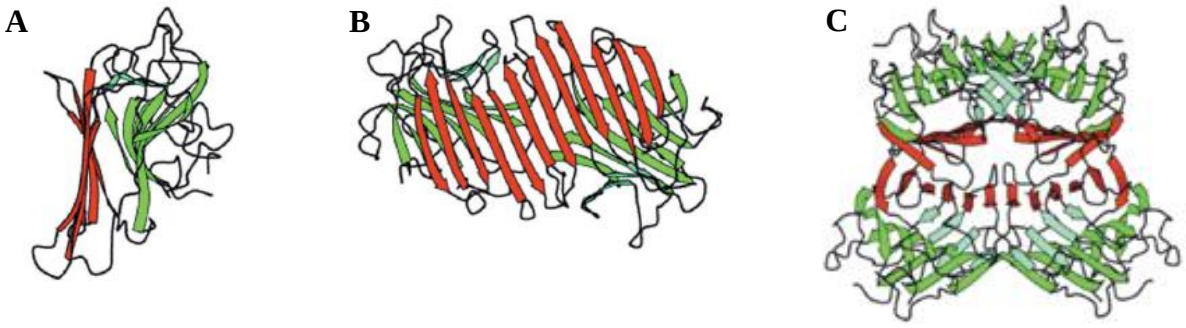


Figure I.2- Representative structure of Concanavalin A lectin. A – Con A fold, tertiary structure of a monomer**. B** – Dimerization in Con A. **C** – Association of two dimers, tetramerization in Con A.
Adapted from Srinivas *et al.*, 2001

The legume lectins commonly consist of two or four 25–30 kDa subunits identical or almost identical, which are commonly single polypeptide chains of about 250 amino acids, and present a tightly bound Ca^{2+} and a transition metal ion, typically Mn^{2+} (Ambrosi *et al.*, 2005; De Hoff *et al.*, 2009).

In fact, these lectin family has very similar primary, secondary and tertiary structure. Almost 60% of their secondary structure comprise of β-strands, connected to each other by loops. The tertiary structure is made up of two anti-parallel β-sheets, a six-stranded flat "back" and a seven-stranded curved "front", connect by a five-stranded β-sheet, this connection may be considered to form the "roof", these structure has been described as to the "lectin fold" (Figure I.2.A), giving the well known the "jellyroll" motif (Srinivas *et al.*, 2001; Ambrosi *et al.*, 2005).

Although there is about 20% conservation in amino acid residues, especially in those involved in the interactions with carbohydrates and in the coordination of metal ions, in particular those responsible for forming of the hydrophobic pocket (De Hoff *et al.*, 2009), small changes in the sequence of the amino acids responsible for the monomer interface may occur. This fact together with the presence or absence of glycosylation seems to affect the association and organization of the monomers, resulting in different dimeric/tetrameric assemblage and consequently in quite different quaternary structures (Srinivas *et al.*, 2001).

In legume lectins with "canonical" quaternary association, for example Con A, the monomers interact extensively, involving a group of around 7 anti-parallel β-sheets that have a generally concave shape, one from each monomer. A considerable portion of the surface is buried in the process, a relatively large surface area, of about 1000 $Å^2$ per monomer (Figure I.2.B). In physiological condition, the association of two dimers gives tetrameric assembly (Figure I.2.C) (Srinivas *et al.*, 2001; Ambrosi *et al.*, 2005).

1.6. Mechanism of action

Carbohydrates are a highly diverse group of molecules and each cell, plasma membrane, or intracellular organelle has a characteristic molecule of these group on the surface. The recognition process to the carbohydrates for a protein is a complex problem and involves a different type of forces and interactions. Since lectins can specifically recognize carbohydrates, these proteins are widely used as model systems for studying these interactions because they are relatively easy to

purify and they cover a wide range of carbohydrate specificities (Loris *et al.*, 1998). This study's is particularly interesting, since it provides the basis for understanding the proteins interactions with the natural ligands (Ambrosi *et al.*, 2005).

The mechanics of carbohydrate recognition by lectins have been studied by several techniques (Ambrosi *et al.*, 2005), which allowed us to understand that although lectins are very different, they have structures with key characteristics shared by this class of proteins (Weis and Drickamer, 1996). In relation to the binding sites for glycans, they are often superficial depressions on the surface of the protein, the combining site appears to be pre-formed, since some conformational change occurring upon binding (Ambrosi *et al.*, 2005).

Regardless of their specificity, all legume lectins have four invariable amino acids residues that are involved in ligand binding, they are: aspartic acid, asparagine, glycine and an aromatic amino acid or leucine (Imberty *et al.*, 1994). However, different legume lectins can show different specificities, the specificity apparently arises from the variability of amino acid residues in other regions of the combined pocket (Ambrosi *et al.*, 2005).

The sugar combining site comprises amino acid residues residing in four loops, designated A, B, C and D (Young and Oomen, 1992). The invariant aspartic acid and glycine belong to A and B, respectively, while the asparagine and the hydrophobic residue are in loop C. The loop D is highly variable in terms of length, sequence and conformation and is responsible for additional interactions, suggesting a correlation between this circuit and the specificity of the lectin (Sharma and Surolia, 1997; Ambrosi *et al.*, 2005).

Although not always directly involved in glycan binding the cations Ca^{2+} and Mn^{2+} (or other transition metal) assist in the positioning of amino acid residues that interact with the glycoside. They are located around 4 Å apart and in close proximity to the sugar-combined pocket. The two invariant residues, aspartic acid and asparagine, are also involved in the coordination of Ca^{2+}. A rare *cisoid*-peptide bond between the critical asparagine and the preceding amino acid, usually alanine, confers on the asparagine residue the proper orientation (Lis and Sharon, 1998; Ambrosi *et al.*, 2005).

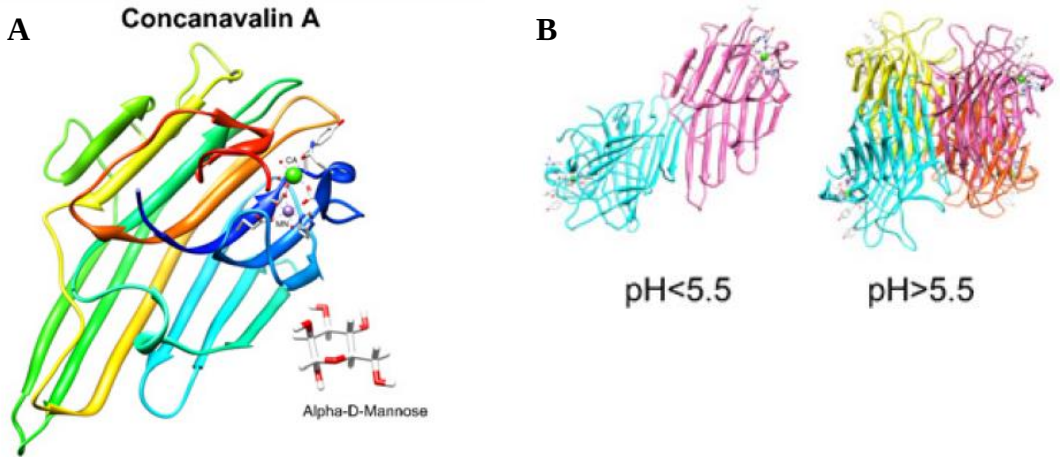


Figure I.3 – Concanavalin A ligand bind representation. A – Con A binding to alfa-D-mannose. **B** – Con A different folding according to the pH.
Adapted from Fu *et al*., 2011

The forces normally present in the binding of carbohydrates to lectins are hydrogen bonds and hydrophobic interactions. The Van der Waals forces contribute to this connection since they are often numerous (Lis and Sharon, 1998). The contact between the carbohydrates and lectins are often mediated by water molecules. The water acts as a molecular link that, because it is a small molecule capable of behaving both as donor and receptor of hydrogen, makes it nearly ideal for this function (Ambrosi *et al.*, 2005).

2. Colorectal cancer and cells glycosylation

2.1. Colorectal cancer

2.1.1. Epidemiology

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second most commonly diagnosed cancer in females. Worldwide in 2012 (**Figure I.4**), the most recent year for which WHO statistics are currently available, occurred 1.361 million new CRC cases and 694,000 CRC related deaths (Ferlay *et al.*, 2015).

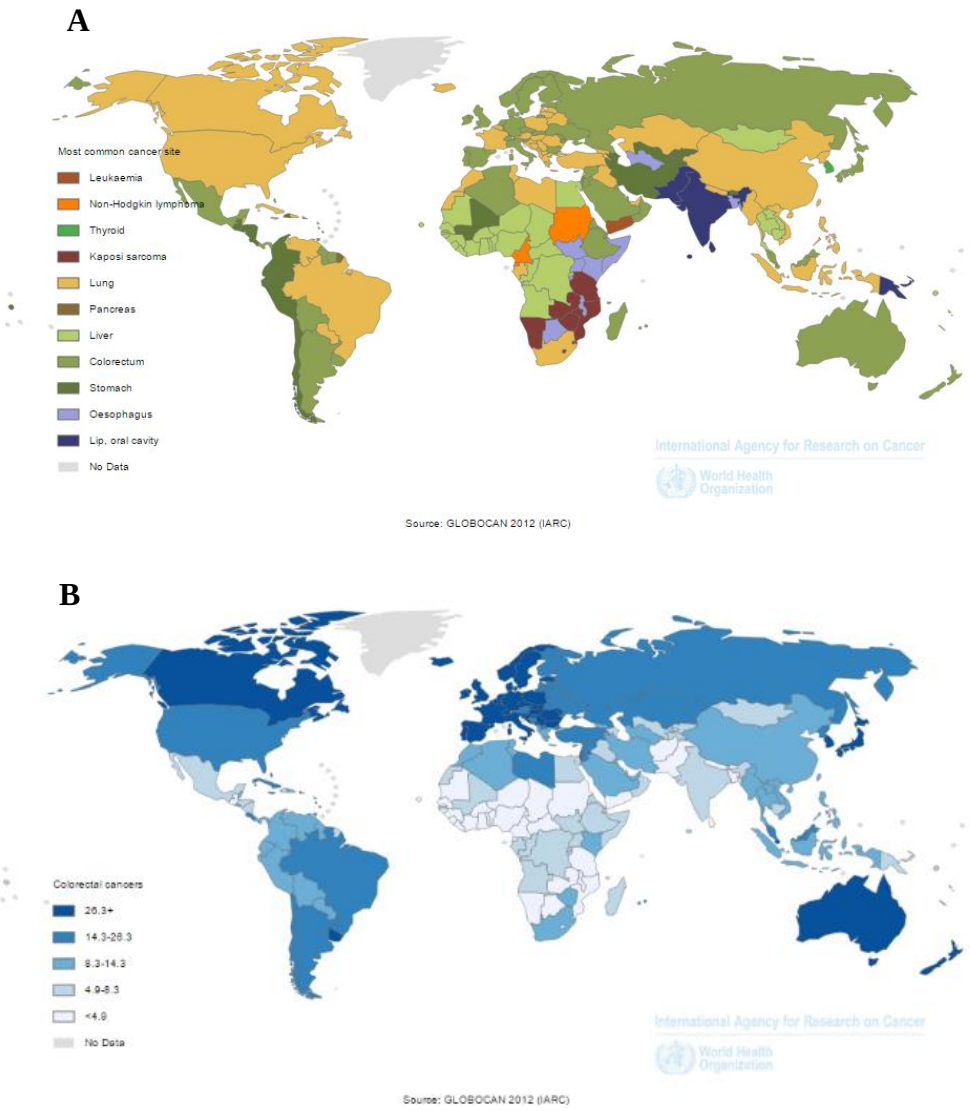


Figure I.4 – **Cancer geographic distribution. A** - Worldwide incidence of all cancer (2012): colorectal cancer represents approximately 10% of all incident cancer, with a large geographic difference in the global distribution, nearly 55% of the cases of CRC occur in more developed regions. **B** - Geographical incidence of CRC variation across the world (2012), high rates in Australia, New Zealand, United States and Western Europe, with the lowest in Africa and some parts of Asia**.**
Adapted from Ferlay *et al*., 2015

There are several risk factors considered to be causally associated with the development of CRC, which can be divided in two categories, the **nonmodifiable risks factors**, that includes age (more than 90% of CRC cases occur in people aged 50 or older), some hereditary factors as, personal history of adenomatous polyps, personal history of inflammatory bowel disease, family history of

colorectal cancer (which represent 25% of the cases (Jorgensen *et al.*, 2015)), adenomatous polyps (FAP) or inherited genetic risk; and **environmental risk factors**, these are also important in the development of this disease but can be modifiable, comprises: diets high in fat, physical inactivity and obesity, cigarrete smoking and heavy alcohol consumption (Haggar *et al.*, 2009).

2.1.2. Diagnosis and treatment

The principal problem of CRC is because this cancer type is often asymptomatic in the first years causing is detection only in stage IV, which reduces the 5-year survival for 13%, when compared to patients who are diagnosed at stages I-II, when the treatments are often curative (Holst et al., 2016). This reveals a need for urgent biomarkers to early diagnostic.

Current treatment is typically by surgical resection and systemic therapy using cytotoxic and target agents and radiotherapy to improve survival (O'neil and Goldberg, 2008; Stein *et al.*, 2011). However, despite the advances in new therapies approximately 30% of patients has recurrence (Marin *et al.*, 2012).

2.1.3. Molecular modification and progression of CRC

This type of cancer results from the progressive accumulation of genetic and epigenetic alterations that lead to the transformation of normal colonic epithelium, lining the large intestine (colon and rectum), to adenomas (Grady and Carethers, 2008; Cooper *et al.*, 2010), and later to adenocarcinoma (**Figure I.5**). In these pathological modifications three main molecular pathways are involved, namely: genetic instability and aberrant DNA methylations by mutations in mismatch repair genes (MMR); mutational inactivation of tumor suppressor genes; and over activation of oncogenic pathways (Grady and Carethers, 2008).

Metastasis represents the final step in CRC progression and involves migration, invasion, resistance, extravasation into the liver and/or lung, and angiogenesis (Valastyan and Weinberg, 2011). This final step depends on two signalling pathways: the vascular endothelial growth factor (VEGF) and the epidermal growth factor receptor (EGFR) pathways (Aghagolzadeh and Radpour, 2016).

Phenotypic changes also occur due to post-translational modifications, namely, modifications in protein glycosylation, more specifically in *N*-glycosylation. *N*-Glycans are of extreme importance in the tumor process, since they are involved in several cellular mechanisms, such as

metabolization, signaling, growth, cell adhesion, cell-matrix interaction, invasion and metastasis (Figure I.5), all of these changes lead to the process progression of the disease (De Freitas Junior and Morgado-Diaz, 2016).

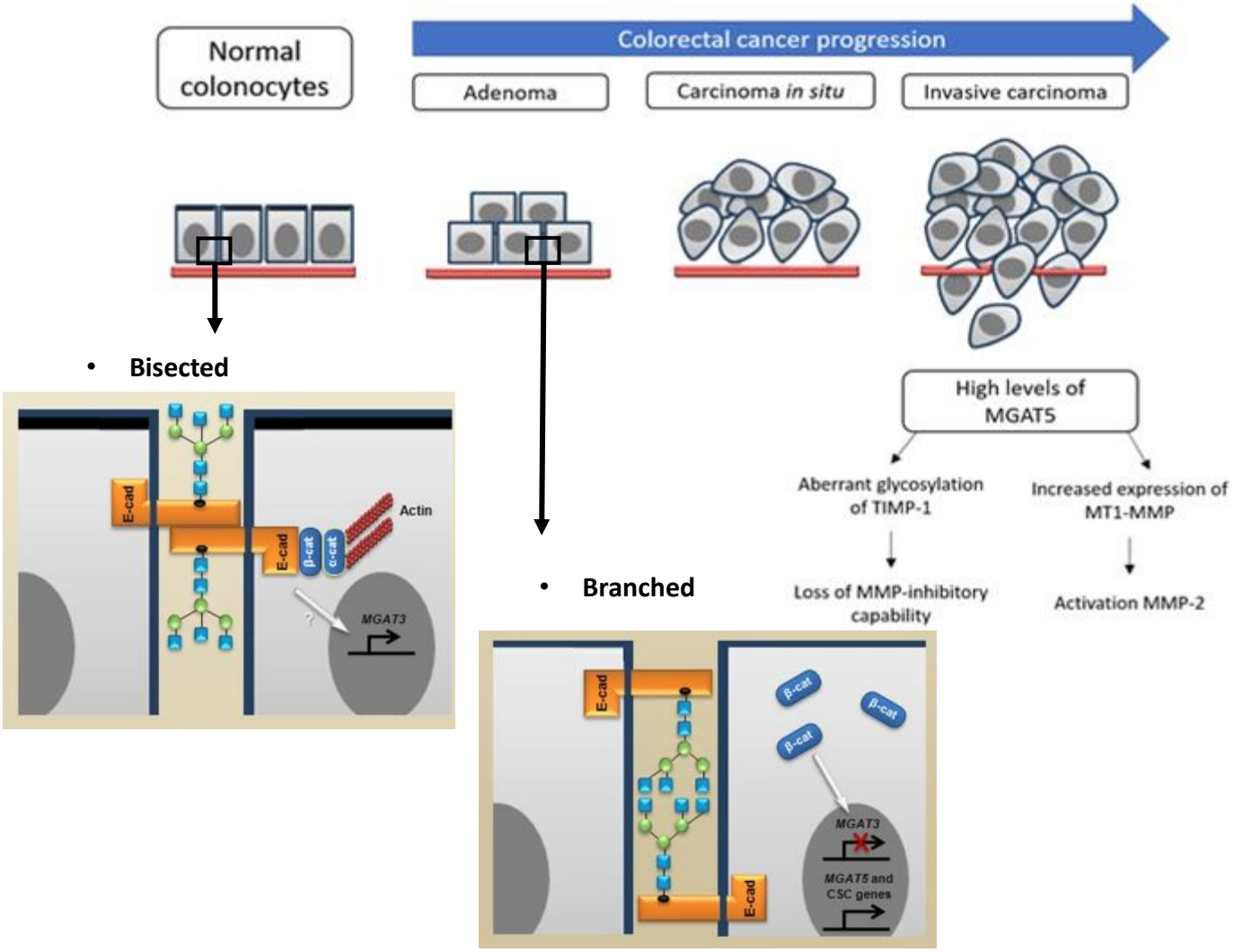


Figure I.5 – Schematic representation of colorectal tumor progression. This is a multistep process where phenotypic changes are involved, namely changes in *N*-glycans. In the early stages, cell adhesion is mediated by E-cadherins, a stable phenotype, however the expression of branched *N*-glycans, induced by *N*-acetylglucosaminyltransferase V (MGAT5), produce an unstable phenotype.
Adapted from De Freitas Junior and Morgado-Diaz, 2016

2.2. Aberrant glycosylation in cancer

2.2.1. Glycosylation overview

Glycosylation is the most frequent post-translational modification of proteins, structurally diverse and more common, and is involved in many essential functions for cell survival (Huang *et al.*, 2014). The combined action of various enzymes (glycosyltransferases and glycosidases) leads to diverse glycan structures, wherein, the *N* and *O* glycosylation are the two glycosylation forms most prominent (Faheina-Martins *et al.*, 2012; Decker *et al.*, 2014). Changes in this process lead to aberration in glycosylation profiles, and seems to be correlate with the development of cancer and other disease states (Sunderic *et al.*, 2015). The lectins due to it glycan specificity can be used as a decoding tool at aberration of cellular glycome.

Glycans are molecules that have the ability to exist in different isomeric forms that yield a variety of branching structures. The complex structures that result from building complex glycans from simple sugars conjugate to a large range of biological molecules such as lipids, proteins, and cell surfaces which generate an array of biological selectivity (Patwa *et al.*, 2010).

Protein glycosylation is the most frequent modification, structurally diverse and more common, and is involved in many functions (Huang *et al.*, 2014), such as, cell-cell signaling, protein stability, protein solubility, interaction of the ligands with the receptors (Yang *et al.*, 2013), growth, differentiation, development, cell division, cell death, the immune response, cancer cell camouflaging, pathogens homing in on host cells, injury and inflammation (Patwa *et al.*, 2010).

Glycosylation is a common post-translational modification of secretory proteins and membrane-anchored lipids which become altered this way when passing through the lumen of the endoplasmic reticulum and Golgi apparatus (Rabinovich and Croci, 2012). Gene products that participate in oligosaccharide biosynthesis account for up to 1% of the translated genome. Of post-translational modifications (PTM), only phosphorylation occurs to a similar magnitude as glycosylation (Patwa *et al.*, 2010).

The machinery of glycosylation is responsible for assembling the abundant and diverse repertoire of glycan structures, which collectively is called glycome, through the synchronized action of glycosidases and glycosyltransferases enzymes. Each of these glycosyltransferases structure uses a single nucleotide and form a specific bond between a monosaccharide and a glycan precursor (Rabinovich and Croci, 2012).

The combined action of the various enzymes leads to diverse glycan structures. These can exist either as free forms or conjugated to proteins and lipids (**Figure I.6**) and include: (1) glycoproteins with complex and branched *N*-linked glycans and/or with *O*-linked glycans that are abundant on mucins (Padler-Karavani, 2014); (2) glycosylphosphatidylinositol (GPI)-anchored proteins (share a common membrane-bound glycolipid linkage structure that is attached to various proteins) (Kinoshita *et al.*, 1997; Padler-Karavani, 2014); (3) glycosaminoglycans (GAGs) either as linear free polysaccharides (such as hyaluronan) or attached to Serine residues of proteoglycans (such as heparin sulphate and chondroitin sulphate) (Esko and Selleck, 2002; Padler-Karavani, 2014); and (4) glycolipids, which consist of glycans linked to ceramide and that include the sialic acid-bearing gangliosides (Maccioni *et al.*, 2002; Padler-Karavani, 2014).

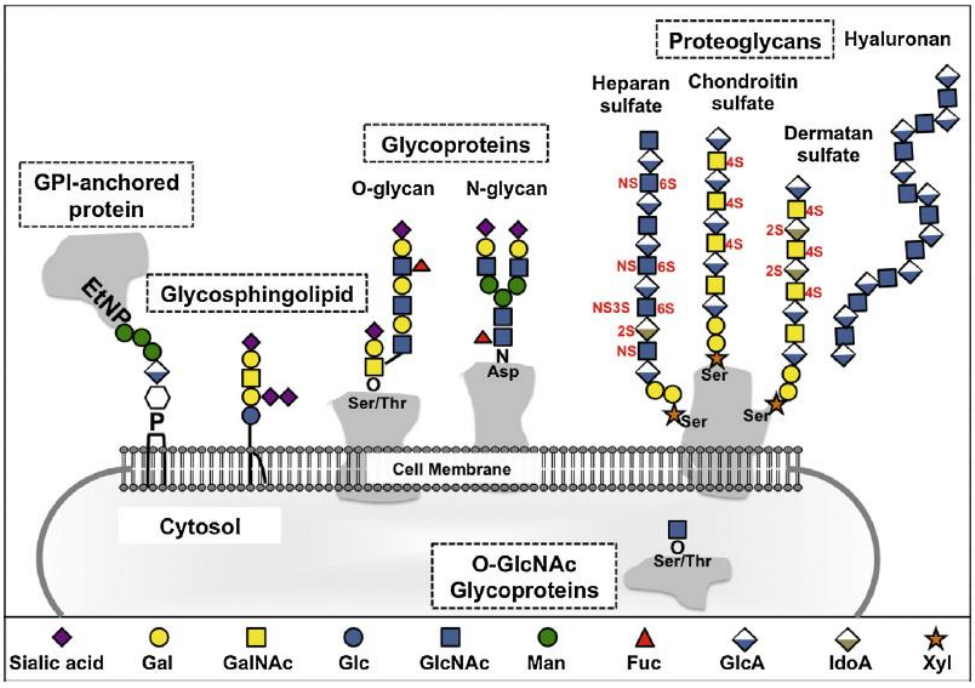


Figure I.6 - Schematic representation of glycoconjugates classes expressed in human cells. Adapted from Padler-Karavani, 2014

The *N* and *O* glycosylation are the two forms of glycosylation most prominent. The *N*-glycans are conjugated to proteins via asparagine residues in a consensus 3 peptide sequence Asn-X-Ser/Thr, where X is any amino acid except proline and the third amino acid can be serine or threonine (Faheina-Martins *et al.*, 2012; Decker *et al.*, 2014). These residues may be enzymatically

released from the protein using *N*-glycosidases (PNGases). The *O*-glycosylation of proteins occurs in both residues serine and threonine, and in this case, there is no specific enzyme comparable to PGNase to remove the intact *O*-glycans link to this end, chemical methods such as β-elimination, are generally used to deliver certain types of *O*-glycan peptides or proteins (Faheina-Martins *et al.*, 2012).

In addition to cell surface glycans, nuclear and cytoplasmic proteins can be modified with *O*-linked *N*-acetylglucosamine (*O*-GlcNAc; conjugated to serine) (Padler-Karavani, 2014). *O*-GlcNAcylation occurs in the cytoplasm and modifies intracellular proteins, regulating their activity together with phosphorylation (Varki and Sharon, 2009).

2.2.2. Abnormal modifications of glycosylation

One of the alterations that occur in cancer cells is in the profile of cell surface (Lahm *et al.*, 2004), particularly, modifications in glycosylation. The associated structural changes that accompany these aberrations can alter the antigenic and adhesive properties of tumor cells, their potential to invade peripheral tissues, and their capacity to metastasize (Hakomori, 2002; Kobayashi *et al.*, 2007; Cho *et al.*, 2010). In fact, the majority of cancer deaths are attributed to the metastatic spread of cancer cells to vital organs rather than to the primary tumor outgrowth (Hauselmann and Borsig, 2014). During malignant transformation, the genetic alteration in the cells results in mutations of proto-oncogenes and tumor suppressor genes, which as a result give rise to tumor clones with different properties (Hanahan and Weinberg, 2011). Malignant cells thereby acquire characteristics enabling them dissociation from tumors, degradation of the extracellular matrix, invasion, adhesion, and metastasis to distant organs (Hauselmann and Borsig, 2014).

In general, a shift from the normal glycosylation pathway leads to altered glycan expression due to one or more of the following changes: (1) under or **overexpression of glycosyltransferases** deregulated at the level of epigenetics (Miyazaki *et al.*, 2004; Kannagi *et al.*, 2008), transcription (Le Marer *et al.*, 1992; Buckhaults *et al.*, 1997; Hatano *et al.*, 2012; Jun *et al.*, 2012), post-transcription (Kim *et al.*, 2013) and/or chaperone (Schietinger *et al.*, 2006); (2) **altered glycosidase activity** (Hakomori, 2002; Kakugawa *et al.*, 2002; Miyagi *et al.*, 2004; Miyagi *et al.*, 2012); (3) **altered expression of glycoconjugate acceptor** together with availability and abundance of the

sugar nucleotide donors (Dennis *et al.*, 1987); (4) **altered sugar nucleotide transporter activity** (Kumamoto *et al.*, 2001); and (5) **improper function of the Golgi structure** (Kellokumpu *et al.*, 2002) where many of the glycosyltransferases are harbored (Moremen *et al.*, 2012).

In this process, a variety of molecules are involved, including various key cell surface proteins that bear complex *N*-glycan arrays, in particular, distinct changes of sialylation and sialylated structures.

The β-galactoside α 2, 6-sialyltransferase (ST6 Gal I) is one of the glycosyltransferases that belongs to sialytransferases family, which are key enzymes in the biosynthesis of sialic acid-containing glycoproteins and glycolipids (Park and Lee, 2013). The ST6 Gal I has been shown to be critical to the development of colorectal cancer (Dall'olio *et al.*, 2006; Swindall and Bellis, 2011; Swindall *et al.*, 2013). Indeed, the upregulation of this sialyltransferase is probably the basis for increased α 2, 6 sialylation seen in CRC cells, positively correlated with metastasis and poor prognosis (Park and Lee, 2013).

2.2.3. Glycosylation modification in cancer

Cancer encompasses a cluster of diseases involving changes in the status and expression of multiple genes, by transforming normal cells into malignant cells, which eventually invade neighboring or distant tissues. These changes confer advantages to tumor cell survival and proliferative potential allowing expression of uncontrolled growth signals, thus making the inhibitory effects of tumor suppressor genes irrelevant.

As previously mentioned, one of the alterations that occurs in cancer is in the profile of cell surface (Lahm *et al.*, 2004), particularly, modifications in glycosylation (**Figure I.7**). Among the most common changes on carcinoma cells are the increase of *N*-glycans and the sialic acid content in the cell surface, the abnormal production of mucin, expression of Lewis x/a structures in glycosphingolipids, and the increased expression of galectins (**Table I.2**) (Hauselmann and Borsig, 2014).

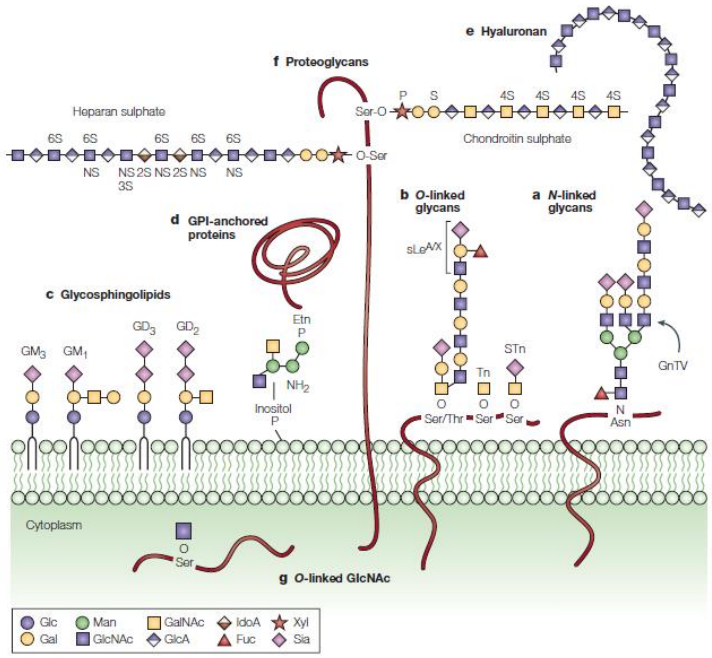


Figure I.7 – Representative glycans involved in tumor progression.

Adapted from Fuster and Esko, 2005

Table I.2 – Common glycan alterations on carcinoma cells.

Structural change	**Carries**	**Biosynthetic basis of structural change**
Increased β1,6-branching (*N*-linked)	*N*-glycans	Increased GnT5
Increased α2,6-sialylation	*N*-glycans, e.g., β-integrin	Increased ST6Gal1 sialyltransferase
General increase in sialylation	Mucins *N*-glycans	Increased sialyltransferase activity
Increased sialyl-Lewis$^{x/a}$	Mucins	Increased FUT7, FUT3, FUT6, ST3Gal6
Decreased di-sialyl-Lewis$^{x/a}$	Mucins, glycolipids	Decreased ST6GalNAc6GlcNAc6ST1
Increased Tn epitopes	Mucins (e.g., MUC1), CD44, β1 integrin, osteopontin	Downregulated T-synthase activity due to Cosmc mutations
Increased sialyl-Tn epitopes		Increased ST6GalNAc1 expression
Increased T antigen (core 1 structure)		Decreased C2GnT2 Enhanced availability og UDP-galactose
Increased sialyl-T antigens		Increased levels of α2,3-sialyltransferase (ST3Gal1)

Adapted from Hauselmann and Borsig, 2014

The structural changes that lead to these aberrations can modify the tumor cells at the level of their adhesion and of antigenic properties, this makes it have the capacity to invade the peripheral tissues and the capacity to metastasize (Hakomori, 2002; Kobayashi *et al.*, 2007; Cho *et al.*, 2010). In fact, metastasize for vital organs is the most responsible for cancer-related deaths (Hauselmann and Borsig, 2014). During malignant transformation, the genetic alteration in the cells results in mutations of proto-oncogenes and tumor suppressor genes, which consequently give rise to tumor clones with different properties (Hanahan and Weinberg, 2011). Malignant cells thereby acquire characteristics enabling their release from tumors, degradation of the extracellular matrix, invasion, adhesion, and metastasis to distant organs (Hauselmann and Borsig, 2014).

Altered branching of N-glycans

The *N*-glycan biosynthesis and their products can be involved in several mechanisms in cancer progression. Some possible mechanism(s) by which these biochemical and structural changes result in the biological outcomes observed include:

- The length of *N*-glycans or the number of *N*-glycosylation sites, these interfere with the cell-cell adhesion mediated by E-cadherin (De Freitas Junior and Morgado-Diaz, 2016);

- The β1,6 branched and bisected *N*-glycan, these modulate cell behavior by interfering in the physical and performance properties of glycosylated adhesion molecules on the cell surface, such as the integrins, and signaling receptors, such as the T-cell receptor and cytokine receptors (Varki *et al.*, 2009; De Freitas Junior and Morgado-Diaz, 2016);

- The upregulation of α2,6-sialylated lactosamine modulates invasiveness and stemness (De Freitas Junior and Morgado-Diaz, 2016), these feature was also responsible for therapeutic resistance in CRC (Park and Lee, 2013);

- An increase in poly-*N*-acetyllactosamine-containing glycans, potentially recognized by galectins, that are preferentially found on this β1–6 branch (Varki *et al.*, 2009);

- Modifications in the cell-surface half-life of growth factor receptors caused by changes in galectin-mediated lattice formation (Varki *et al.*, 2009);

- The increased outer-chain polyfucosylation and sialyl Lewis^x production (potentially recognized by the selectins) (Varki *et al.*, 2009);

- A general biophysical effect of the branching itself on membrane protein structure (Varki *et al.*, 2009).

The increased size of tumor cell–derived glycopeptides is due an increase in β1–6 branching of *N*-glycans (**Figure I.8**), these results from enhanced expression of UDP-GlcNAc:*N*-glycan GlcNAc transferase V (GlcNAcT-V). The viral and chemical carcinogenesis may be responsible for the increased transcription of gene *MGAT5*, that affect modifications in expression of this enzyme GlcNAcT-V (Varki *et al.*, 2009; Hauselmann and Borsig, 2014).

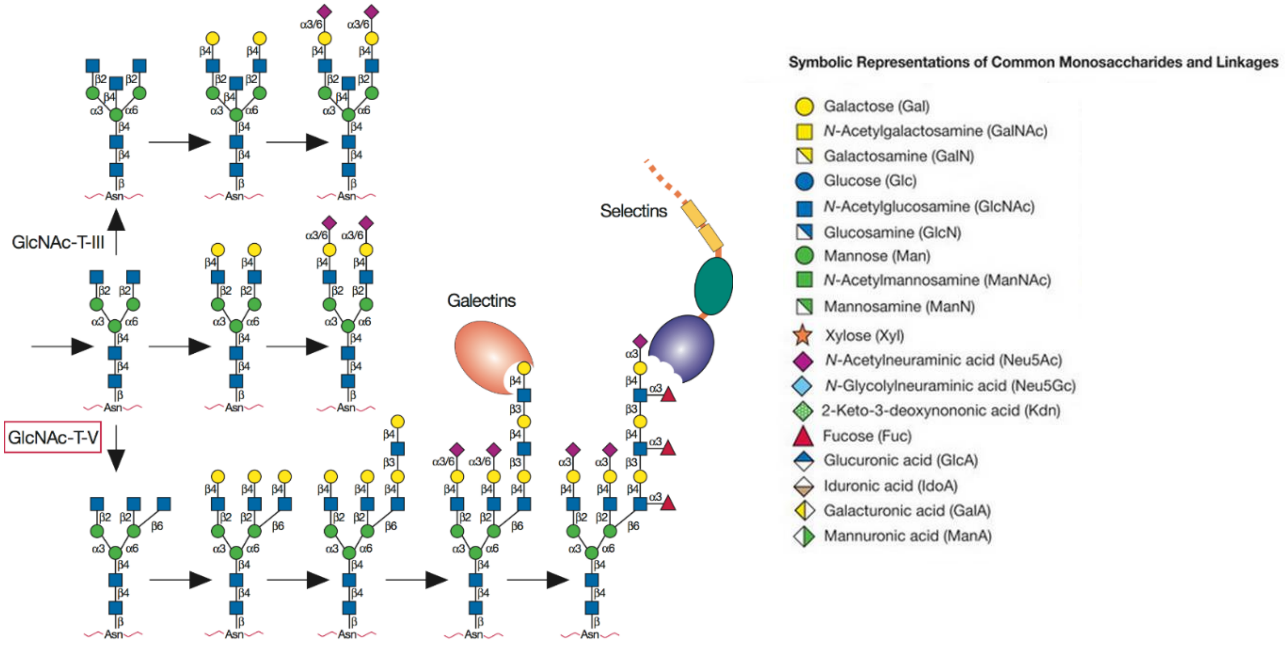


Figure I.8 – The GlcNAc transferase-V (GNT-V) increase activity can explain the increased size of *N*-glycans that occurs upon transformation.
Adapted from Varki *et al*., 2009

Altered branching of O-glycans

Abnormal *O*-glycans expressed by cancer cells have functional importance in cell adhesion, invasion and metastasis (Baldus *et al.*, 2004). In particular, an incomplete elongation of *O*-glycans in **mucins** (**Figure I.9**), that are large glycoproteins with a "rod-like" conformation, which carry many clustered glycosylated serines and threonines in tandem repeat regions (Varki *et al.*, 2009), lead to the expression of shortened carbohydrate structures, such as the **Tn** (αGalNAc-*O*-Ser/Thr), **sialyl-Tn** (αNeuAc2,6-αGalNAc-*O*Ser/Thr) or **TF** (βGal1,3-αGalNAc-*O*-Ser/Thr) antigens (Osinaga, 2007).

These structures, are very important since they have particular features, important in cancer biology. They can be excellent diagnostic markers, they are related to the aggressiveness of the tumor, the more antigens are the more aggressive is the carcinoma (for example, TF antigens interacts with galectin 3, and this interaction is involved in the cellular adhesion of some cancer cells) (Osinaga, 2007) and can be processed by T cells and presented by the major histocompatibility complex eliciting thereby specific immune responses (Werdelin *et al.*, 2002).

A number of factors can influenced the mucin-type *O*-glycosylated antigens expression, for example: the availability of functional ppGalNAc-Ts, the amino acid sequence environment, post-translational modifications of the peptide substrates, and/or the availability of glycosyltransferases (Osinaga, 2007).

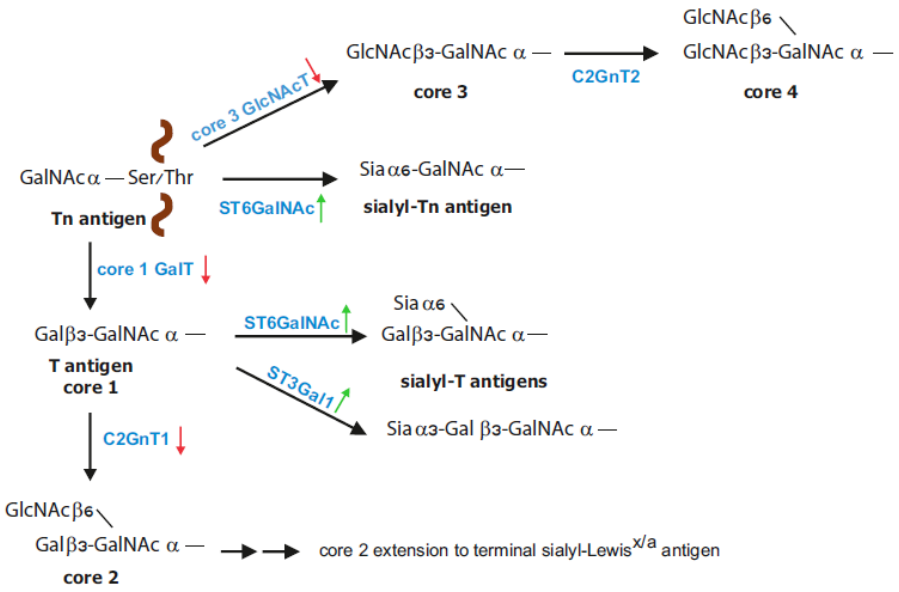


Figure I.9 – Biosynthesis scheme of *O*-glycans.
Adapted from Hauselmann and Borsig, 2014

Altered sialic acids expression

The surface properties of cancer cells are different than those of normal cells and one of the things that contribute to that phenotypic change are the sialic acids. Sialic acids (Sia) are nine-carbon backbone α-keto acidic sugars, that at physiological pH has the carboxylate groups negatively charged. Modifications in Sia level, linkage, distribution and increased activity of sialyltransferases are associated with various aspects of malignant transformation and promote metastic potential (Padler-Karavani, 2014).

As previously mentioned, the most common aberrant glycosylation in cancer are in *N*-glycans, like, the increased activity of the β1-6GlcNAc branching enzyme, GlcNAc-TV or MGAT5, that lead to larger and more branched *N*-glycans providing additional acceptors for terminal sialylation. These changes together with the increase expression of sialyltransferases, contributes to cell surface sialylation and metastatic potential.

On the other hand, the aberrant *O*-linked glycosylation can also lead to increased sialylation, the overproduce mucins (that are heavily glycosylated glycoproteins, characterized by dense clusters of *O*-glycans) produce cancer-associated to mucin-type *O*-glycans and tend to be truncated due to a shift in the normal enzymatic machinery and are usually highly sialylated and less sulphated (Padler-Karavani, 2014).

3. Cellular death mechanisms and lectins as antitumoral agents

3.1. Cell death mechanisms

Cell death can be classified into: "accidental" and "regulated". **Accidental cell death** (ACD) is caused by severe insults, including physical, chemical and mechanical stimuli, is insensitive to pharmacologic or genetic interventions of any kind and includes cell death of the necrotic type. On the other hand, **regulated cell death** (RCD, include **programmed cell death** (PCD), comprise apoptosis, autophagy, necroptosis and lysosomal-mediated cell death (Kreuzaler and Watson, 2012)), can be altered by means of pharmacologic and/or genetic interventions targeting the key components of such a machinery, and involves a genetically encoded molecular machinery (Galluzzi *et al.*, 2015).

3.1.1. Accidental cell death

Necrosis is a kind of death in which cells undergo an increase in cell volume, chromatin aggregation, disruption of cytoplasm, loss of plasmatic membrane integrity and subsequent cell disruption. During necrosis, cellular contents are released, causing damage to surrounding cells and an inflammatory reaction on the local (Ziegler and Groscurth, 2004). More recently, regulated necrosis or necroptosis has also attracted much attention as a specific signaling pathway amenable to therapeutic targeting (Galluzzi *et al.*, 2015).

3.1.2. Programmed cell death

Apoptosis is a complex but highly defined cellular program of cell death, tightly regulated by multi-step pathway that is responsible for cell death not only during development, but also in adult multicellular organisms, in which it partly controls cell balance. It is characterized by cell shrinkage, chromatin condensation, and nuclear and cell fragmentation. These features result in the formation of apoptotic bodies that are then engulfed by neighboring phagocytic cells (Cotter, 2009).

The mechanism of apoptosis requires the interplay of numerous factors such as caspases (Saraste and Pulkki, 2000). Fourteen human caspases are known and six of them (caspases -2, -3, -6, -7, -8, -9, -10) are involved in apoptosis. Caspases cleave substrates with aspartate residues leading to condensation, nuclear fragmentation and externalization of membrane phospholipids. This will signal that these cells are to be phagocytosed by macrophages (Nicholson and Thornberry, 1997; Boatright and Salvesen, 2003).

p53 is often regarded as the "guardian of the genome" (Lane, 1992) as it exerts tumor suppressor capabilities through central coordination of a regulatory circuit that monitors and responds to a variety of stress signals, including DNA damage, abnormal oncogenic events, erosion of telomeres, hypoxia and ribosomal stress (Kruse and Gu, 2009; Luo *et al.*, 2013; Hao and Cho, 2014). However, under normal physiological conditions, p53 is maintained at low intracellular concentrations through its negative regulator, Mdm2, which targets the degradation of p53 by ubiquitin-dependent proteasome (Carvajal and Manfredi, 2013).

The activation of apoptosis can be initiated in two different ways: the extrinsic pathway (cytoplasmic) or the intrinsic pathway (mitochondrial), and these two ways are interconnected. The extrinsic pathway is triggered by the binding of specific ligands to death receptors (DR), which allows the activation of the caspase cascade. Effector caspase activation by initiator caspases leads to cleavage of the substrate starting the morphological changes and leading to cell death. These substrates are proteins that belong to various functional classes including: apoptosis signaling proteins (eg IAPs, Bcl-2); protein kinases (eg FAK, PKC); structural/cytoskeletal proteins (eg gelsolin, lamin); cell repair proteins (PARP, ATM); and cell cycle proteins (eg p21, Rb, p27). Most proteins are inactivated following caspase cleavage and some are activated.

The intrinsic pathway is activated by intracellular or extracellular stress as lack of growth factors, DNA damage, hypoxia, oncogene activation and as a result of extrinsic pathway activation.

Mitochondria are the principal mediators of such cell death stimuli. When death signals reach mitochondria, they lead to collapse of mitochondrial inner membrane potential and rupture of the organelle. Consequently, pro-apoptotic proteins are released, which leads to loss of cellular homeostasis, disruption of ATP synthesis and increased production of reactive oxygen species.

Caspase activation by DR is suppressed by FLIP, a master anti-apoptotic regulator and resistance factor that suppresses tumor necrosis factor-α (TNF-α), Fas-L, and TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis, as well as apoptosis triggered by chemotherapy agents in malignant cells. Induction of the DR pathway, which results in the activation of caspase-8 can lead directly to the activation of caspase-3 without the need for mitochondrial damage. Alternatively, it may involve Bid, resulting in the potential loss of mitochondrial membrane and release of cytochrome c into the cytoplasm. Cytochrome c in the presence of ATP and Apaf1 activates caspase-9, which results in the activation of caspase-3. The anti-apoptotic molecules Bcl-x_L and Bcl-2 protect against loss of mitochondrial membrane potential that is induced by pro-apoptotic molecules such as Bax and Bak.

Autophagy is an evolutionarily conserved and adaptive process under genetic control. This occurs in response to metabolic stress that results in the degradation of cellular components (Lum *et al.*, 2005). In many tumors, the autophagic cell death mechanism is usually hiper-activated and inhibition can lead to increased death or survival depending on the cancer type (Kreuzaler and Watson, 2012).

During autophagy, cytoplasmic portions are encapsulated by cell membranes, resulting in structures called autophagosomes. These will merge with lysosomes and the contents of autophagosomes will be degraded by lysosomal hydrolases (Kelekar, 2005). The genes that codify proteins (ATG genes) involved in autophagosome formation are tightly regulated (Klionsky, 2007), showing the importance of the complex. The Atg proteins, including ATG5, ATG7, ATG4B, and LC3, are involved in the fusion of the secretory lysosomes with the plasma membrane and subsequent formation of the ruffled border. The LC3 promotes the phagosomes fusion with lysosomes leading to a rapid acidification (Reggiori, 2012).

Necroptosis is a death mechanism that has necrotic phenotype but can be regulated. The necrotic phenotype can be explained by the absence of caspase activation, particularly caspase-8.

This cell death type is dependent on the activity of the death domain (DD)-containing kinase RIP1, which is essential for the execution of the necroptotic programme (Kreuzaler and Watson, 2012).

3.2. Some lectins with antitumoral effect

Since lectins are excellent proteins capable of cell recognition by glycans binding, some plant lectins are used as simple tumor recognition tools, in diagnosis and prognosis, to differentiate malignant tumors from benign and the degree of glycosylation associated with metastasis (Gupta *et al.*, 2010). In addition, some lectins also possess antitumor activities, via targeting **programmed cell death** (PCD) (Fu *et al.*, 2011).

Several plant lectins such as Mistletoe lectins (MLs) and Ricin have been well-studied to possess antiproliferative and apoptosis-inducing activities toward cancer cells (De Mejia and Prisecaru, 2005). Besides, other lectins such as ConA and PCL can result in autophagic cell death after internalization or binding certain sugar-containing receptors on the surface of cancer cells (Lei and Chang, 2007).

Following are some examples of plant lectins that lead to programmed death via targeting apoptotic and autophagic pathways in various types of cancer cells.

3.2.1. Molecular mechanisms of Mistletoe Lectins (MLs)

The Mistletoe lectins (MLs) are divided into three distinct types, namely, ML-I, ML-II and ML-III with therapeutically activity. These lectins show different specificity, being the ML-I with D-galactose specificity and ML-II and ML-III preferentially bind to *N*-acetylgalactosamine (Bantel *et al.*, 1999). MLs lectins are well-studied type II ribosome inactivating proteins (RIPs II), inhibin the intracellular protein synthesis via interacting with 28S ribosome, also they have antiproliferative and apoptosis-inducing mechanisms (**Figure I.10**) in cancer cells (Bantel *et al.*, 1999; Seifert *et al.*, 2008).

The ML-I can induce apoptosis by **extrinsic apoptotic pathway** activating and subsequently to caspase 8, through TNF-family death receptors in determining cancer cell death. In some types of cells cancer ML-I induces apoptosis by the breakdown of mitochondrial membrane potential leading to cytochrome c release and increase of the levels of reactive oxygen species (ROS) and caspase-3 activation independently of p53, but apoptosis-associated factor-1 (Apaf-1)-dependent pathway (Hostanska *et al.*, 2003).

The ML-II can activate MAPK signaling, implicated in ERK and p38, this can modulated the signaling pathways that lead to anti-proliferative and apoptosis inducing activities (Fu *et al.*, 2011). This protein can also induce apoptosis via activating SAPK/JNK and p38 pathways, as well as via inhibiting ERK1/2 pathway in human monoblastic leukemia U937 cells (Pae *et al.*, 2001).

3.2.2. Molecular mechanisms of proteins with legume lectin domains (Con A)

Proteins that possess legume lectin domains have a wide range of biological functions such as antitumor, anti-viral, and anti-fungal activities. Concanavalin A, that can be extracted from Jack bean (*Canavalia ensiformis*) seeds, is a mannose/glucose-binding lectin legume with apoptosis-inducing activities (**Figure I.10**), that can be mediated by **mitochondria mediated apoptotic pathway** (Fu *et al.*, 2011; Yau *et al.*, 2015), mitochondrial membrane potential collapse, cytochrome c release and caspase 9/3 activation.

This lectin has been widely studied since the observation of cell death mechanism. One of the molecular mechanism that Con A can induce cellular death is by autophagic via BNIP3 (pro-apoptotic protein) in hepatoma cells (Hannigan and Gorski, 2009). Con A binds to mannose residue in cells membranes and is internalized to the mitochondria surface resulting a reduction in the mitochondrial membrane potential. LC3-II protein formation is related to BNIP3 and beclin-1 proteins induction. The BNIP3 determine the state of permeability of the mitochondrial pores (Chang *et al.*, 2007). In these process were also detected double-layer vesicles and acidic vesicular organelle (Li *et al.*, 2010).

Another study has also shown Con A capacity to induce apoptosis regulated by autophagy in HeLa cells (Roy *et al.*, 2014). Usually, the phosphatidyl-inositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway is activates and the Raf/MEK/ERK pathway is inhibited in cancer cells. The Con A treatment in HeLa cells reduce Akt phosphorylation and mTOR activity, showing that Con A has the capacity to downregulated the PI3K/Akt/mTOR pathway and upregulated the MEK/ERK pathway (Roy *et al.*, 2014), leading to autophagy in these cells.

3.2.3. Molecular mechanisms of Galanthus nivalis agglutinin (GNA) family

Polygonatum cyrtonema lectin (PCL), a mannose/sialic acid-binding lectin from GNA family, has been study for its role in cancer cell death targeting PCD pathways (**Figure I.10)**, as apoptosis and autophagy. These lectin can induce these two types of cell death and they are connect with

each other to participate in leading to cancer cell death via promoting a mitochondria-mediated ROS-p38-p53 pathway (Liu *et al.*, 2009), as well as via blocking Ras-Raf and PI3K-Akt pathways (Wang *et al.*, 2011).

Polygonatum odoratum lectin (POL), another lectin from GNA family has also been described with capacity to induce cell death by apoptosis and autophagy by targeting epidermal growth factor receptor-mediated Ras-Raf-MEK-ERK signaling pathway. Also, these lectin shows apoptosis selectivity for lungs cancer cells but not for healthy lung cells (Yau *et al.*, 2015).

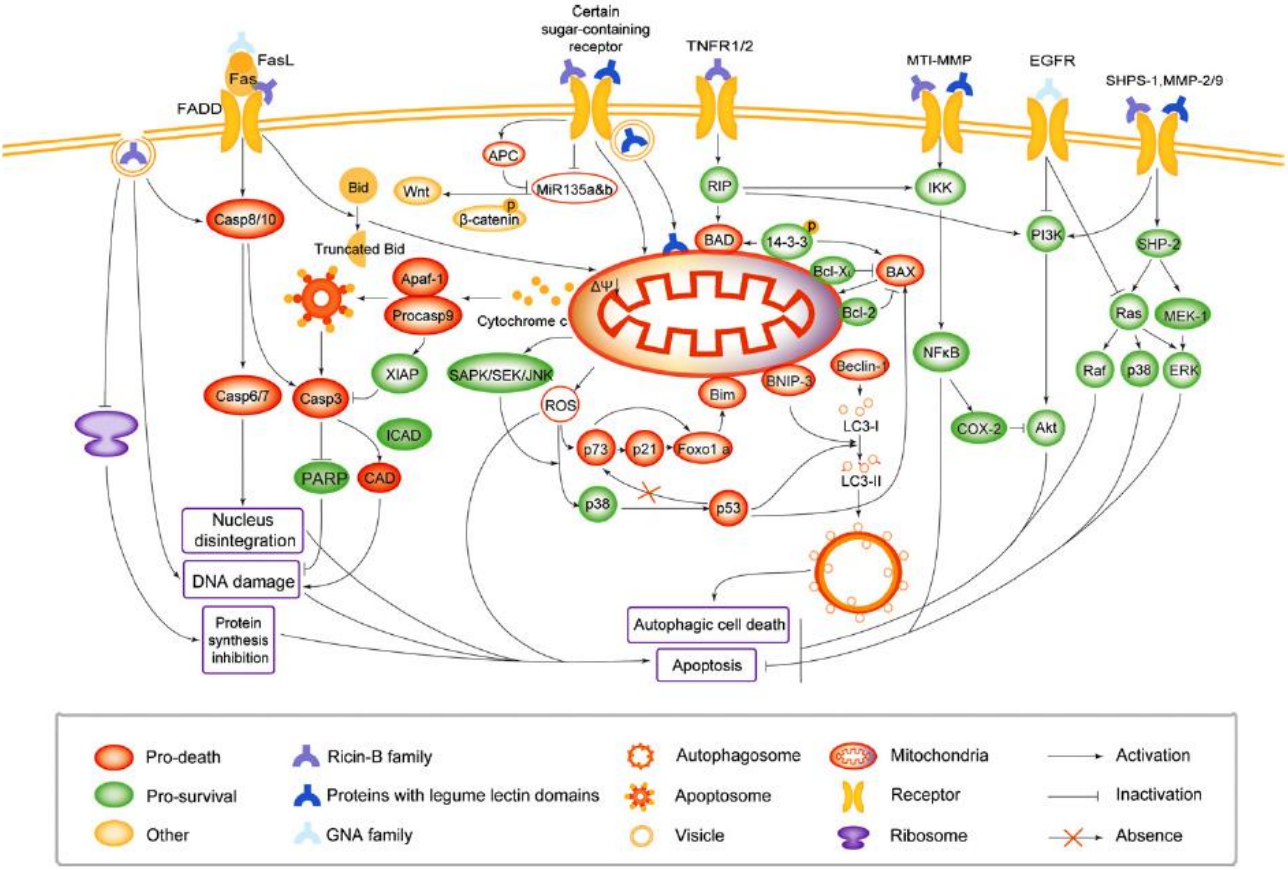


Figure I.10 - Plant lectins induce cancer cell death via targeting programmed cell death (PCD) signaling network.

Adapted from Fu *et al.*, 2011

After the general review of the different cancer cell death mechanisms by plant lectins described so far, we can conclude that there are three hypotheses for lectins to induce death in cancer cells: directly interfering in the ribosome of cancer cells, to inactivate it; after endocytosis, selective binding to certain organelles, such as mitochondria; and binding to receptors that contain sugars

on the cells surface, this being of paramount importance since these receptors are often exclusive of cancer cells, making this binding quite selective (Fu *et al.*, 2011).

The study of glycosylation profiles changes can be carried out by using lectins, since these have determinant regions that allow unique specificity for a particular glycan and generally do not interfere with the protein structure. They are also able to interact with several molecules of biological fluids and cell surface receptors, acting as decoding of information exchanged between molecules, cells or organisms. This possible application of lectins, opens the way for the early diagnosis and treatment, where lectins may be used as therapeutic agents (**Table I.3**) or as drug vehicle.

Table I.3 – Examples of lectins with antitumor potential known, the cell lines affected by each lectin and some of the known mechanisms involved.

Lectin	Cancer Cell Lines Affected	Effector Mechanism(s)
Galectins	Epidermal keratinocytes, 1299 lung cells, fibroblast cells, thyroid cell, colon cells	T-cell binding, specific integrin binding, Ca^{2+}-calpaincaspase-1 pathway
C-Type Lectins	SW1116 colorectal cells	Le glycan recognition, perforin granzyme pathway, TRAIL and FAS lingand binding
Annexins	Melanoma cells, lung cells	NF-κB signal transduction pathway, Ras-Raf-MAPK pathway, p53 apoptotic pathway
Sialic acid binding *Haliotis discus discus* lectin (HddSBL)	Hep3B hepatocellular cells, SW480 colorectal cells, A549 and H1299 lung cancer cell line cells	Bcl-2 down-regulation
Polygonatum odoratum lectin (POL)	A549 lung cells, L929 murine fibrosarcoma cells	Akt-mTOR pathway, Fas mediating apoptotic pathway, TNFα enhancement
Mistletoe lectin	Hepatocarcinoma cells, breast cancer cells, NALM-6 acute lymphoblastic leukemia cells, glioblastoma cells, hepatomacarcinoma cells, peripheral blood mononuclear cells, A253 epidermoid cells	Wnt signaling miR-135a and b, NK-mediated cell lysis, interleukin mRNA activation
Concanavalin A (ConA)	A375 and B16 melanoma cells, fibroblast 3T3 cells, colorectal cancer cells	Mitochondrial apoptotic pathway, caspase induction

Adapted from (Yau *et al.*, 2015).

The antitumor effect of lectins in a cell line of human colorectal cancer, HT29 cells, has been demonstrated and several mechanisms of action have been proposed. These include binding to cell membrane (Nunes, 2014), cytotoxicity (*Amaranthus caudatus*) and tumor inhibition (*Artocarpus integrifolia*), as well as inhibition of proliferation (*Sauromatum venosum*, *Arbutus unedo*, *Juniperus oxycedrus var. badia*) (Castillo-Villanueva and Abdullaev, 2005) and MMP-2 and MMP-9 activity inhibition (Nunes, 2014).

4. New lectins in Mediterranean flora as therapeutic tools for cancer

Since ancient times the plants are widely used due to it's curative and preventive capabilities and it's high content of bioactive principles, being a valuable source of therapeutic agents. The WHO estimates that traditional medicine, mostly plant-derived drugs, is responsible for the health care of 80% of the world population. Given these facts, it is no surprise that the study of potential medicinal plants and their bioactive compounds have, today, huge relevance.

Here are some examples of species of Mediterranean flora that were studied due to it's high content in bioactive compounds, thus giving them preventive and healing properties in several pathologies. Generally, the bioactivities described have been attributed to various classes of active principles, with special relevance to the phenolic compounds and alkaloids. The study of lectins has been relegated to the background. For it's antitumor mechanism of action and by the fact that the plants are an excellent reservoir of lectins, we consider to be promising it's evaluation in species with pharmacologic properties already described.

4.1. *Arbutus unedo* L.

Arbutus unedo L. is a small evergreen tree that belongs to the Ericaceae (Carcache-Blanco *et al.*, 2006). This strawberry tree thrives on the Iberian Peninsula and in the Mediterranean basin, as well as in other regions with hot summers and mild rainy winters. The spherical fruits with a diameter of about 2–3 cm are red and tasty only when fully ripe. The fruits are edible but usually processed before consumption (Oliveira *et al.*, 2009).

The **fruits** contain several antioxidant molecules namely phenolic compounds (e.g. anthocyanins, gallic acid derivatives, tannins and flavonoids), vitamin C, vitamin E and carotenoids (Oliveira *et al.*, 2009). Their use is widespread in traditional medicine as antiseptic agents, laxatives

and diuretics. It has been well established that a diet high in fruits reduces the risk of cardiovascular diseases, cancer and other diseases mediated by oxidative stress (Carcache-Blanco *et al.*, 2006).

The phytochemical studies of the **leaves** showed that leaf extracts contain several phenolic compounds, like tannins, flavonoids, phenolic glycosides (Oliveira *et al.*, 2009), as well as proteins like lectins (Ribeiro *et al.*, 2012), among others. They are used as astringent, diuretic, urinary anti-septic, antidiarrheal, depurative and more recently in the therapy of hypertension, diabetes and in the treatment of inflammatory diseases. Still, a recent study prove that leaf extracts exhibited excellent free radical scavenging activities and also potent in scavenging superoxide radical. This could be beneficial to prevent and treat illnesses and improve overall health of patients (Oliveira *et al.*, 2009).

4.2. *Corema album* L.

The *Corema album* (Ericaceae) is an evergreen dioecious shrub which rarely exceeds 1 m in height. *Corema album* is endemic to the Atlantic coast of the Iberian Peninsula (Alvarez-Cansino *et al.*, 2010). Its fruits are fleshy, more or less spherical berries (5-8 mm diameter) white or pink gently (Brunetti-Pierri *et al.*, 2008; Mohamed *et al.*, 2009). The edible berries are an important source of nutrients and phytochemicals, since they have been described as a good source of phenolic acids and flavonoids (Hariharan *et al.*, 2008).

The **fruits** of *Corema album* have high quantities of phenolic compounds which include phenolic acids, of flavonol glycosides, proanthocyanidins and anthocyanins. Its wealth in these bioactive principles gives them many properties beneficial to health, such as prevention of urinary tract infections, neuroprotection, decreased risk of cardiovascular disease and anticarcinogenic action (Kunnumakkara *et al.*, 2012).

The **leaves** are also rich in flavonoids and other secondary metabolites (Leon-Gonzalez *et al.*, 2014). A recent survey shows that extract from the leaves of *Corema album* L. have cytotoxic activity against the HT29 human colon adenocarcinoma cell line. Two dihydrochalcones, 2',4'-dihydroxydihydrochalcone and 2'-methoxy-4'-hydroxydihydrochalcone, have been isolated from the leaves of *C. album*, both compounds showed higher cytotoxicity than the positive control 5-fluorouracil (5-FU) (Leon-Gonzalez *et al.*, 2013). Another study, also shows that *Corema album* leaves are rich in proteins like lectins (Nunes, 2014).

4.3. *Juniperus* L.

The genus *Juniperus* L. belongs to the Cupressaceae family and condenses about 70 species, which are distributed in the Northern Hemisphere (Gutman and Kessler, 2006). These plants have an extensively history of use in global folk medicine for various disorders, such as common colds, urinary and kidney infections and dermatological disorders. Many biological activities have been reported for *Juniperus* sp. These include anti-inflammatory, diuretic, antiseptic (bacterial and fungal), anthelmintic, hypoglycaemic, hypotensive, abortifacient, anticonceptive, antiviral, anticancer, antioxidant, anticholinesterase and analgesic properties (Tavares *et al.*, 2012).

The *Juniperus oxycedrus* L., known as prickly juniper, plum or juniper juniper-red berries, is a shrub or small tree native throughout the Mediterranean region, Morocco and Portugal. *J. oxycedrus* L. includes three subspecies: subsp. oxycedrus, subsp. *macrocarpa* (Sm). Ball and subsp. *badia* (H. Gay) Debeaux. The fruit *J. oxycedrus* have cytotoxic activity to many types of human tumor cells and antioxidant activity (Helman *et al.*, 2014).

The *Juniperus* species represent a rich source in bioactive compounds, such as, catechins, procyanidins, flavonoid derivatives, flavones and biflavones, present in the leaves of the various species of *Juniperus*, that show to have cytotoxic effects against tumor cells of different origins (Tavares *et al.*, 2013; Taviano *et al.*, 2013).

The bioactive principles present in Mediterranean flora have pharmacotherapeutic properties in various diseases, particularly in cancer. Their potential use in antitumor therapy has been investigated in recent years, and various *in vitro* and *in vivo* assays have been developed with positive effects. However, in the majority of the studies the bioactivities described have been attribute to various classes of active principles, with special relevance to the phenolic compounds and alkaloids.

Until now the study of lectins has been relegated to a second plan. There are few studies relating the antitumor activity of these plants with the presence of lectins, and those that exist require additional testing to gain strength. So, for its antitumor mechanism of action and by the fact that the plants are an excellent reservoir of lectins, we consider to be promising its evaluation in species with pharmacologic properties already described.

Recently, a study in protein extracts of four Mediterranean flora species, *Juniperus oxycedrus* var. *oxycedrus*, *Juniperus oxycedrus var. badia*, *Arbutus unedo* and *Corema album*, showed a

potential relevance for antitumoral activity. The lectins constituents of these extracts reveal recognition for glycosylated epitopes at the level of HT29 cells membranes from colorectal cancer. The binding of these lectins to HT29 cells and the exhibition of antitumor activity of these species allow us to study its responsibility on the antitumor activity as well in the mechanism involved in the cellular death. Therefore, after lectin recognition of the glycan specificity, they can be used as a probe for the recognition of the tumor cell or for therapy (Nunes, 2014).

5. Aims

The specificity of certain lectins for carbohydrates allowed their use in research, in order to recognize cells of different etiologies (red blood cells and tumor cells) using glycosylated receptors (glycolipids, glycoproteins and proteoglycans). An increasing number of studies have demonstrated that carbohydrates on the cell surface are modified during malignant transformation, yielding differences in cells that can promote cellular invasion, favoring the metastatic process (Xu *et al.*, 2000). Studies with some lectins showed that they exhibit a greater binding on cancer cells compared to normal cells of the same type (Nunes, 2014).

There is an increasing need for new therapeutic strategies for cancer in general, and also for colorectal cancer in particular, one of the most prevalent forms of cancer. Knowing that plants are excellent lectin reservoirs and that many lectins have already well described antitumor mechanisms, this project aims to select one of four species of Mediterranean endemic flora, including *Juniperus oxycedrus* var. *oxycedrus*, *Juniperus oxycedrus* var. *badia*, *Arbutus unedo* and *Corema album*, with hemagglutination activity already described. Using the selected species, we will purify and study the antitumor mechanism of one or more lectins, in human HT29 colorectal cancer cells, and will characterize the proteomic profile.

This project is divided into four tasks: (1) screening of species with antitumor activity in HT29 cells; (2) polypeptide characterization of selected species extract(s); (3) purification of lectins from selected species; and (4) final evaluation of antitumor mechanism of purified lectins in HT29 cells.

Chapter II – Materials and Methods

1. Screening of plant species with antitumor activity in HT29 cells

1.1. Material

1.1.1. Biological material

Plant material

To initiate the assays was used leaves from four plant species, particularly, *Juniperus oxycedrus* subsp. *oxycedrus*, *Juniperus oxycedrus* subsp. *badia*, *Arbutus unedo* and *Corema album*.

Human material

The HT29 cell line of colon carcinoma, from the species *Homo sapiens*, was a kind gift from Dr. Peter Jordan (Instituto Nacional de Saúde Doutor Ricardo Jorge), originally obtained from ATCC.

1.2. Methods

1.2.1. Human colon carcinoma cell line HT29 and cell culture

All HT29 cell manipulations regarding culture maintenance, trypsinization, plating and extract incubations were carried out under sterile conditions in a laminar flow chamber. Cell culture maintenance was performed in cell incubators at 37 °C under a humidified atmosphere of 5% (v/v) CO_2.

The HT29 human cell line of colorectal carcinoma was grown in RPMI medium (Roswell Park Memorial Institute - Gibco, Life Technologies) medium supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Sigma), 2 mM Glutamine 0.5% (v/v), Penicillin (2 x 10^4 IU/mL) and 34 mM Streptomycin (Pen-Strep) and then was filtered in sterile microfilter (GVS Polyethersulfone Membrane 0.22 µm). The stock culture was maintained in flasks.

When cells reached 80% of confluence, the medium was removed and trypsin (TrypLE Express - Gibco, Life Technologies) added for 15 min at 37 °C. The medium was then removed and centrifuged at 500 *g*, for 5 min, leaving only enough volume for cells to grow again. After centrifugation, the supernatant was removed and 1 mL of RPMI added to the pellet and homogenized. An aliquot was withdrawn from the cell homogenate and trypan blue was added to count cells in the Neubaumer's chamber. Cells were counted and the necessary volume calculated for the subsequent assays, followed by dilution with RMPI and plating.

1.2.2. Measurement of cell death and viability

The evaluation of cell viability and cell death were performed using the MTS metabolism and lactate dehydrogenase assays, respectively. These assays were performed in parallel. 5 x 10^3 cells per well, in 96-well plate, were incubated with 100 µg/mL of each total proteins extracts of the species under investigation and with saline containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$ as a control, for 48 and 72 h.

1.2.2.1. Measurement of general cell death by lactate dehydrogenase (LDH) assay

General cell death was evaluated using LDH Cytotoxicity Detection Kit^{PLUS} (Roche Diagnostics GmbH, Germany). The lactate dehydrogenase release assay measures membrane integrity as a function of the amount of cytoplasmic LDH released into the medium. The assay is based on the reduction of NAD by LDH. Reduced NADH is then used in the conversion of a tetrazolium dye to a colored product that is measured by a spectrophotometry at 490 nm.

After incubation of 100 µg/mL of total protein extract in HT29 cells, 50 µL of culture supernatant was collected from each well and added to a new 96-well plate. Then the plate was incubated with 50 µL of assay substrate (prepared by mixing 1,11 µL of catalyst for each 50 µL of Dye Solution), for 10 to 30 min, at room temperature, protect from light. The absorbance was measured at 490 nm, with 620 nm reference wavelength using a Model 96 microplate reader (GLOMAX by Promega).

1.2.2.2. Measurement of cell viability with MTS metabolism assay

Cell viability was evaluated with CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega), using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS). In brief, this is a homogeneous, colorimetric method that detects viable cells in proliferation, cytotoxicity or chemosensitivity assays. The CellTiter 96®AQueous Assay is composed of solutions of MTS and an electron coupling reagent (phenazinemethosulfate, PMS). MTS is bioreduced by cells into a formazan product that is soluble in tissue culture medium. The conversion of MTS into the aqueous soluble formazan product is accomplished by dehydrogenase enzymes found in metabolically active cells, and is directly proportional to the number of viable cells in culture.

After removed the incubation media that will be used for LDH assay, the remaining incubation media was replaced by 120 µL MTS/PMS mix (100 µL of culture media, 19 µL of MTS and 1 µL of PMS). Then the cells were incubated at 37 ºC for 1 h. Changes in absorbance were measured at a wavelength of 490 nm, using a Model 96 microplate reader (GLOMAX by Promega).

2. Polypeptide characterization of *Arbutus unedo* leaf extracts

2.1. Material

2.1.1. Biological material

Plant material

Was used leaves of *Arbutus unedo*. The final extracts were stored in -80 º C freezer in aliquots.

Human material

For this work was used membranes of cellular line HT29 of colon carcinoma.

For the hemagglutination assays was used rabbit erythrocyte from *Probiológica*.

2.2. Methods

2.2.1. *Arbutus unedo* total protein leaves extraction

The total protein extract of leaves from *Arbutus unedo* were obtained by an optimized method of extraction with buffer containing 5% (v/v) glycerol and 1% (v/v) Triton X-100, described by Silva and Souza (Silva and Souza, 2009). This method has a greater extraction power compared to other methods, since the solution contains Triton X-100, a non-ionic detergent which dissolves cell membranes and solubilizes proteins, while maintaining their native conformation. The original concentration of 4% (v/v) Triton was adjusted to 1% (v/v) to minimize cell lysis during assays performed in erythrocytes or HT29 cells.

Leaves from *A. unedo* were powdered under liquid nitrogen, after which was added 0.25 g of polyvinylpolypyrrolidone per 0.5 g of fresh leaves, according to Silva and Souza. A protease inhibitor tablet containing 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), bestatin, pepstatin A, E-64, leupeptin and 1,10-phenanthroline (Sigma) was added at the beginning of the extraction procedure (1 tablet per 10 mL extraction buffer). The total proteins were extracted by adding 1 mL of extration buffer (Tris–HCl 0.5 M, pH 8.0, containing 5% (v/v) glycerol, 0.1 M KCl and 1% (v/v)

Triton X-100) per 0.5 g fresh leaves. The slurry was centrifuged at 14,000 *g* for 30 min at 4 ºC, the supernatant was filtered with Miracloth (Calbiochem) and desalted through PD-10 columns previously equilibrated in Tris–HCl 0.05 M, pH 7.5 buffer, containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$.

2.2.2. Determination protein concentration by Bradford Method

A subsequent determination of total proteins by Bradford Method (Bradford, 1976) was performed. These method is a colorimetric assay that involves the interaction of the dye Coomassie Brilliant Blue G250, dissolved in phosphoric acid and methanol (Bradford Reagent), with the basic or aromatic amino acid side chains, promoting a blue coloration by the stabilization of the dye anionic form, being more intense the greater the amount of protein present in the sample. This colour change can be quantified by measuring the absorbance at a wavelength of 595 nm, helping to determine the protein concentration. This assay is fast and highly reproducible.

Protein concentration was establish using a calibration curve in 96-well plates. For the calibration curve was used Bovine Serum Albumin (BSA) as standard protein with concentrations between 0 and 25 µg and in triplicates. The samples of total extract protein were also applied in triplicates. Finally, the Bradford reagent (BioRad) was added in all wells and incubated for 15 min, at room temperature, protect from light. Absorbance readings were measured at 595 nm (Software KinetiCalc – KC 4).

2.2.3. Polypeptide profile evaluation by electrophoresis

In order to evaluate the polypeptide profile of total protein extract, electrophoretic separations were carried out on polyacrylamide gels under denaturing and reducing conditions (SDS-PAGE R), according to the methodology described in Santos (Santos *et al.*, 1997).

This technique is based on the amphoteric property of proteins and separation by molecular weight. The proteins are subjected to an electric field, where its mobility, from the cathode to the anode, depends in the first instance of it's load and it's size, and therefore, a migration according to their molecular weight, implies that all proteins have a similar charge density/mass. This is achieved by the addition of denaturing agents to the sample as SDS (anionic detergent), which will impart an overall negative charge to the polypeptides. The SDS has the ability to form a strong complex SDS/polypeptides due to their apolar character, connecting to micellar form to protein

apolar regions, and being its ionic area exposed to the solvent (Robyt and White, 1990). The binding of SDS to protein promotes two distinct effects, the dissociation of protein oligomers and the disruption of the protein secondary structure. Also, saturation of the protein with SDS leads to camouflage protein load, and the migration in the gel became to be completely dependent on their molecular weight (Plummer, 1987). The use of β-mercaptoethanol promotes the reduction of possible disulphide bonds (Robyt and White, 1990).

The separation gel consisted of an acrylamide matrix of 17.5% (w/v), 0.1% (w/v) bisacrylamide, 375 mM Tris-HCl buffer pH 8.8, 0.03% ammonium persulfate (PSA), 0.03% (v/v) Tetramethylethylenediamine (TEMED). The concentration gel consist of 5% (w/v) acrylamide matrix, 0.13% (w/v) bisacrylamide, 125 mM Tris-HCl buffer pH 6.8, 0.1% (w/v) ammonium persulfate (PSA), 0.05% (v/v) Tetramethylethylenediamine (TEMED). A 16 cm x 18 cm x 0.75 mm gels are normally used. After assembly of the vertical system and its sealing was verified, the constituent solutions of the gel were added with a micropipette. The separation gel was initially applied up to 2 cm from the top of the glass, which was covered with a thin layer of butanol, avoiding the formation of undesirable surface air bubbles in order to favor uniform polymerization. After polymerization, all the butanol was removed from the vertical system, then was added the concentration gel, after insertion of the comb into the mold, to form the filling wells. The gel, after polymerization, were placed in the electrophoresis chamber (HOEFER SE 600 Ruby from Amersham Biosciences) and was added the electrophoresis buffer solution (200 mM Glycine, 25 mM Tris-Base, 0.02% (w/v) SDS) in order to cover the electrodes. The electrophoresis was performed under reducing conditions using sample buffer contained 100 mM β-mercaptoethanol. All sample were denatured at 100ºC for, 4 min, followed by application on the gel, where it was also applied 3 μL of molecular weight marker (BioRad), ranging from 10 to 259 kDa. The gel was subjected during running to an electric current of 70 milliamperes (mA) and a potential difference of 220 V (Pharmacia Gene Power Supply EPS 600) per unit of gel. The run finished when the bromophenol blue (front-line marker) reached the end of the gel.

2.2.4. Polyacrylamide gel staining by silver nitrate

For the staining of the polyacrylamide gel by silver nitrate ($AgNO_3$), the method of Blum was applied (Blum *et al.*, 1987). These method is based on the ability of proteins have to bind to silver ions (Ag^+). It is believed that complexation of Ag^+ ions with amino groups, occurs in an alkaline

environment and in the presence of sulphur from the methionine and cysteine residues. The matrix of the separated proteins gel is saturated with Ag^+ ions. These ions preferentially bind to basic amino acids with characteristics of surface proteins from the gel matrix and, ions that do not bind to any amino acid have to be removed.

The staining procedure was initiated by immersing the gel in a fixing solution (50% (v/v) methanol, 12% (v/v) acetic acid and 0.02% (v/v) formaldehyde) and placed on an orbital shaker with low stirring for 20 min, there being no inconvenience of overnight development. The fixing solution was discarded and the gel was washed three times, for 10 min each, with wash solution in 50% (v/v) ethanol. After removal of the washed solution, a pre-treatment was carried out for 1 min by immersing the gel in a 0.02% (v/v) sodium hyposulfite solution, after which the gel was washed with distilled water, for 20 s each, three times. The staining solution (11 mM $AgNO_3$, 0.03% (v/v) formaldehyde) was added, allowing contact with the gel for 10 min, then washing twice with distilled water for 20 seconds each. After a development solution (566 mM Na_2CO_3, 0.02% (v/v) formaldehyde, 16 μM $Na_2S_2O_3$) was added during the time required for the appearance of the bands. At the end the staining reaction was stopped by immersion the gel in stop solution (50% (v/v) methanol, 12% (v/v) acetic acid). The gels were conserved after dehydration in cellophane sheet.

2.2.5. Lectins detection

After protein extraction, we confirmed the presence of lectins with hemagglutination assay using rabbit erythrocytes. Also, we verified the specificity of these lectins against a panel of selected sugars.

The blood group is defined by antigens (glycoproteins and glycolipids) present in the erythrocyte cell membrane. These genetic markers are species-specific and differ in immunogenicity. A set of two or more alleles at a locus constitutes the blood system. The general term used to describe the visible agglomeration of erythrocytes is hemagglutination. Lectins possess the ability to induce cell clumping phenomenon and the presence of this family often detected using hemagglutination assay (Ribeiro *et al.*, 2012). Many lectins recognize differences between blood group antigens.

2.2.5.1. Preparation of rabbit trypsinized erythrocyte solution at 4% (v/v)

The total protein extract obtained as described in 2.2.1. was tested against a rabbit erythrocyte solution at 4% (v/v).

After receiving the blood collected by the *Probiológica*, a previous centrifugation, 1,100 *g* was carried out, for 6 min, in order to separate the pellets (sediment) from the respective plasma. Washing of the globules was followed by saline (40 mL of saline:5 mL of blood) three times. For each wash was performed a centrifugation (1,100 *g*, for 6 min, 13 ºC) in order to separate the globules, discarding the supernatant**.** The washed and sediment erythrocytes (the pellet occupies a volume of approximately 4 mL) were resuspended in 96 mL of saline to which trypsin (Sigma) at the final concentration of 1% (w/v) was added and incubated 1 h at 37 ºC with stirring. Further centrifugation (1,100 *g*, for 6 min, 13 ºC) was performed to remove the supernatant, and three new washes were carried out as before. Finally, the sedimented erythrocytes were resuspended in 96 mL of saline solution and left with a solution of 4% (v/v) erythrocytes.

These erythrocytes thus treated were divided and stored at 4 ºC for use in hemagglutination tests. The erythrocyte solution was considered within the validity while it did not show erythrocyte hemolysis.

2.2.5.2. Hemagglutination assay

The hemagglutination activity was determined by serial dilution method on microplates (microdilution). In this assay, a serial dilution of the total protein extracts is performed in saline (NaCl 0.9% (w/v)), in a geometric progression of ratio 3, followed by incubation with rabbit trypsinized erythrocytes at 4% (v/v). The hemagglutination activity is expressed as Hemagglutinating Units (H.U.), considering as the lowest concentration of protein that still expresses hemagglutination activity on the erythrocyte. This activity is determined by the ratio between the concentration of total protein and tested 3^{n-1}, with n being equal to the last dilution which still exhibits hemagglutination activity (Ribeiro *et al.*, 2012).

$$H.U. = \frac{\text{total protein}}{3^{n-1}}$$

Assay performed on 96-well plate with U-bottom. A volume of 70 µL of saline was deposited in each well of the plate. The protein sample was deposited in the volume of 70 µL, containing 50-

100 µg of protein in saline solution (containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$), in the first well and a serially dilution 1:3 of the sample was started. After that, 70 µL of the erythrocyte solution, 4% (v/v), was deposited in all wells and the protein was diluted 1:3. A negative control (-) of saline and a positive control (+) of concanavalin A (1 mg/mL) were always present. The plate was incubated without stirring at 37 °C, for 30 min.

A negative result is revealed by the formation of a red blood cell precipitating nucleus surrounded by a clear colourless supernatant. A positive result is manifested by the adherence of the erythrocytes to the bottom of the plate, forming a coating and sometimes may also exhibit a certain retraction of the erythrocyte coating.

2.2.5.3. Determination of hemagglutination activity inhibition by carbohydrates

The definition of the glycoside specificity of a lectin can be achieved by assays of inhibition of hemagglutination activity with different monosaccharides and polysaccharides (Sharon and Lis, 1990).

The specificity tests of carbohydrates, were carried out as described by Ribeiro and colleagues (2012). Four hemagglutination units (H.U.) of the protein in study were incubated with a battery of 16 carbohydrates, each diluted at the ratio of 1 to 3. Carbohydrates used in the test were: glucose (0.1 M); glucosamine (0.1 M); *N*-acetyl-D-glucosamine (0.1 M); galactose (0.1 M); galactosamine (0.1 M); *N*-acetyl-D-galactosamine (0.1 M); lactose (0.1 M); mannose (0.1 M); raffinose (0.1 M); D-fucose (0.3 M); melezitose (0.1 M); methyl-α-glucopyranoside (0.1 M); methyl-α-mannoside (0.1 M); sucrose (0.1 M); *N*-acetylneuraminic acid (0.1 M) and *L*-fucose (0.3 M).

This assay was performed in microplate with the U-bottom, where a volume of 70 µL of saline was added in each well of the plate. Each sugar (70 µL) was successively diluted 1:2 in saline. After that, the protein sample (4 U.H.), in a 70 µL volume was deposited in each well. It was allowed to incubate for 1 h, at room temperature. Finally, 70 µL of erythrocyte solution at 4% (v/v) was deposited in all wells, being the diluted sugar 1:3. It was allowed to incubate again for 1 h, at room temperature. Negative and positive controls were always performed, as described in 2.2.5..

A positive result is revealed by the formation of a red blood cell precipitating nucleus surrounded by a clear colourless supernatant. In these cases, there were inhibition of hemagglutination, meaning that the sugar, bound to the protein revealing specificity and the protein was unable to bind to the glycosylated receptors of erythrocytes, minimal inhibited concentration

(m.i.c.). In these cases, the minimum sugar concentration that still causes inhibition of agglutination is noted. A negative result is manifested by the adherence of the erythrocytes to the bottom of the plaque, forming a coating meaning that the protein did not bind to sugar.

2.2.6. Polypeptide binding to HT29 cell membranes

The total protein extract from *Arbutus unedo* leaves, was incubated with HT29 membranes for assess the binding potential of proteins to glycosylated cell membranes receptors.

Membranes isolation

The HT29 cell stored in cryotubes at -80 °C, were thawed rapidly at 37 °C, and the cell suspension was pooled into a conical tube, to which was added 10 volumes of HES buffer (20 mM HEPES, pH 7.4, 250 mM sucrose), for washing and elimination of the cryocrystallization medium, by centrifugation at 750 *g*, for 10 min, at 21 °C (Beckman J2 -21M/E, Rotor JA 20.000). The supernatant was discarded and the wash operation was repeated for one more time, the pellet obtained was resuspended in HES buffer, added protease inhibitor cocktail (EDTA-free, Roche). The cell lysis was performed by cryolysis, where the cells were subjected to a freeze-thaw (4x), for 30 min at -20 °C, associated with sonic sonication for 20 min. A centrifugation at 960 *g* was carried out for 10 min, at 4 ° C, and the pellet was discarded at the end. Finally, the supernatant was ultracentrifuged at 10,000 *g*, for 45 min at 4 °C (Beckman J2 -21M/E, Rotor JA 20.000). The pellet, containing the cell membranes, was resuspended in 2 mL of saline solution (0.9% (w/v) NaCl) containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$. Subsequently, the pellet was divided into aliquots containing 1 mg of protein, after determination of the protein content by the Bradford method, according to the procedure described in 2.2.2..

Protein extract HT29 cells membranes incubation

A 1000 µg of HT29 membranes previously solubilized in 3.5 mL of saline containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$, were incubated with 1.4 mg of total protein extract also in saline containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$, during 35 min, at 25 ºC, with gentle shaking. After this time, was realized a sequence of three centrifugations, at 12,100 *g* and 4 ºC, during 8 min, in order to remove the proteins that does not bind to membrane. In the end of each centrifugation the supernatant was discard and 10 volumes of saline containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$ were added to pellet. After the last centrifugation, the pellet was solubilized in saline containing 2 mM

$CaCl_2$ and 2 mM $MgCl_2$. In parallel, a blank assay was performed, replacing the total protein extract by saline containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$.

2.2.6.1. Polypeptide binding protein to HT29 membranes by SDS-PAGE R evaluation

To evaluated the binding of total protein extract to HT29 membranes was performed an electrophoretic technique by SDS-PAGE R as previous described in 2.2.3.. It was applied 13 µg of HT29 membranes, 50 µg of total protein extract and 13 µg of HT29 membranes incubated with total protein extract. To visualized the gel was performed a silver nitrate staining, as previous described in 2.2.4..

3. Purification of Arbutus unedo lectins

3.1. *Arbutus unedo* lectins purification by Q-Sepharose

The first step for lectins purification from the total protein extract of *A. unedo* leaves, was with an anion exchange column of Q-Sepharose gel (quaternary ammonium salt from Pharmacia).

A 20 mL of total protein extract (obtained as described in 2.2.1.) in buffer A (Tris-HCl 50 mM, pH 7.5, 2 mM $CaCl_2$ and 2 mM $MgCl_2$), was injected in a Q-Sepharose column, previously equilibrated in buffer A. During the chromatography procedure, in AKTA apparatus (FPLC - fast protein liquid chromatography), the unadsorbed proteins on the column were pooled and stored at -80 º C, for subsequent assays. Elution of the adsorbed proteins from the column was done by stepwise gradient: 0-20% step gradient in 4 column volumes (CV), 20-30% in step gradient 4 CV, 30-40% 3 CV, with later linear gradient of 40-60% 2 CV and finally step gradient 60-100% in 3 CV of buffer B (Tris-HCl 50 mM, pH 7.5, 1 M NaCl, 2 mM $CaCl_2$ and 2 mM $MgCl_2$), taking into account that a column volume was 6 mL. The gradient was formed automatically by mixing buffer A with buffer B.

The chromatographic fractionation was carried at a flow rate 1 mL/min of buffer solution and with continuous reading of absorbance at 280 nm. The eluates were collected by monitoring of the chromatogram. After elution, the protein quantification by the Lowry method was performed.

3.1.1. Determination protein concentration by Lowry method

The Lowry method (Lowry *et al.*, 1951) is one of the most sensitive procedures for protein quantification, however it is influenced by numerous interfering substances. Bensadoun and Weinstein (Bensadoun and Weinstein, 1976) developed a technique based on the traditional method, which allows to overcome some of these interfering factors.

The Lowry method is based on the ability of the protein to react with copper in an alkaline medium (biuret reaction), forming a complex capable of reducing the Foli-Ciocalteau reagent (phosphomolybdenum and phosphotungsten), resulting in a compound of intense blue color that absorbs at 750 nm. By a previous precipitation with trichloroacetic acid (TCA), associated to the solubilizing effect of the sodium deoxycholate, a protein precipitate is obtained, which ensures reproducibility in the quantification of proteins, eliminating the interfering materials.

A calibration curve was constructed with BSA solutions of concentrations between 0 and 35 µg. To the BSA solutions, as well as to the sample solutions, water was added to make up 250 µL. After 50 µL of 1% (w/v) sodium deoxycholate and 1 mL of 10% (w/v) TCA was added. After 10 min incubation, the samples were centrifuged for 5 min at 10,000 *g*, the supernatant was discarded.

The previously formed protein precipitate was resuspended in 1 ml of reagent C (reagent B (4% (w/v) of copper sulphate pentahydrate) diluted 1:100 in reagent A [2% (w/v) of sodium carbonate, 0.4% (w/v) sodium hydroxide, 0.16% (w/v) sodium tartrate and 1% (w/v) SDS]. After 10 min, 100 µL of Folin-Ciocalteau reagent diluted in water (1:1) were incubated in the samples, in the dark, for at least 30 min to a maximum of 3 h, after which the absorbance was read at 750 nm in a spectrophotometer Shimadzu UV-2100.

3.1.2. Lectins detection

After protein concentration determination, hemagglutination assays were carried out to check for the presence of lectins in all samples, including the unadsorbed fraction, according to the assay described in 2.2.5.2.. After that, sugar specificity was determined only for the sample which showed hemagglutination activity, the unadsorbed fraction, called from now on “unadsorbed Q-Sepharose” (Unds Q-Seph), using the method described in 2.2.5.3..

At the same time, the polypeptide profile of the same samples was evaluated through a polyacrylamide gel electrophoresis. The procedure was the same as described in 2.2.3., and the amount of protein samples applied on the gel was: 40 µg for the total protein extract, 20 µg for the

unadsorbed sample to Q-Sepharose and 7 µg for all eluates. To visualized the gel was performed a silver nitrate staining, as previous described in 2.2.4..

3.2. Unadsorbed Q-Sepharose lectins purification by affinity columns

Proteins that did not bind to the Q-Sepharose (unadsorbed Q-Sepharose fraction) and exhibit hemagglutination activity, were pooled and applied on three different affinity columns. These columns were chosen based on the sugar affinity assays.

Affinity chromatography is a technique used to purify compounds, such as proteins, that have the ability to non-covalently and reversibly bind specific molecules, known as ligands. Before loading the sample, the gel was equilibrated with an adequate buffer (binding buffer), after what the sample was loaded, in the same buffer. After washing the column with the same buffer, the lectins bound to the matrix were eluted by the elution buffer containing a specific sugar for each lectin (determined by the previous inhibition of hemagglutination activity assays). The eluates were monitoring at 280 nm for detected the eluted protein. The eluted lectin, after protein quantification, was visualized by electrophoretic profile and characterized concerning the lectin profile by hemagglutination activity (Pohleven *et al.*, 2012).

3.2.1. Protein separation by affinity columns

The three columns used were selected based on the previous sugars inhibition tests performed on total protein extract and unadsorbed Q-Sepharose fraction. Thus, the first column in which the unadsorbed Q-Sepharose sample was tested was *N*-acetyl-D-glucosamine (Vector), followed by D-galactose (Pierce) and finally, *N*-acetyl-D-galactosamine (Sigma).

Each column was prepared with 500 µL of the respective affinity gel. The column protocol was started by washing with 10 volumes of distilled water three times to remove all the preservation solution. The equilibrium washing of the column was followed by 10 volumes, twice, with the respective binding buffer, for *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine columns was used 10 mM TBS, pH 7.5, and for D-galactose (Pierce) column was 0.1 M TBS, pH 7.2. Once the column was equilibrated, the Q-Seph Unds fraction (previously equilibrated in the column buffer) was loaded. After entering the sample, 10 volumes of the equilibration buffer were applied to remove proteins which did not bind or which were non-specifically bound to the column. Elution of the proteins bound sugar matrix of each column was followed with elution buffer, which is equal

to the elution buffer with addition of the respective sugar for each column, for *N*-acetyl-D-glucosamine column the sugar was 0.4 M of *N*-acetyl-D-glucosamine, for D-galactose column the sugar was 0.2 M D-galactose and for the *N*-acetyl-D-galactosamine column the sugar was 0.3 M *N*-acetyl-D-galactosamine. Elution was carried out by eluates absorbance measure at 280 nm.

After all eluates were collected, NAP 10 columns containing the Sephadex G-25 gel, were used for sugar removal and exchange of the buffer with saline containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$ and by ultrafiltration systems. After the affinity column, hemagglutination activity was determined, as described in 2.2.5.2., in unadsorbed fraction and on eluates. Subsequently, an SDS-PAGE-R gel was performed in order to evaluate the polypeptide profile of the samples with hemagglutination activity.

3.3. Unadsorbed Q-Sepharose lectins purification by Mono S

Before proceeding with Mono S column, it was checked whether the lectins present in the unadsorbed Q-Sepharose sample did not lose hemagglutination activity at low pHs. For this, the hemagglutination activity was tested after subjecting the sample to low pH.

The sample was incubated in citrate buffer 20 mM, at pH 2.0, 2.5, 3.0, 3.5 and 4.0, for 30 min, at 4 ºC. The NAP-10/PD-10 columns were used to exchange the buffer. After this time, the samples were centrifuged at 10,000 g, for 10 min, in order to remove the proteins which had undergone acid hydrolysis. The supernatant was collected and the samples were passed to 50 mM Tris-HCl, pH 7.5 buffer through NAP-10/PD-10 columns. At the end, the samples were transferred to saline solution and hemagglutination assays were performed**,** as described in 2.2.5.2..

After determining that the sample did not lose the hemagglutination activity at low pHs, we continued with lectins purification, present in the unadsorbed Q-Sepharose sample, using the Mono S column, a strong cation exchanger (Resource S).

A 8 mL of total unadsorbed Q-Sepharose fraction in buffer A (20 mM phosphoric acid and NaOH, pH 2.0, 2 mM $CaCl_2$ and 2 mM $MgCl_{2)}$ was injected Mono S column, previously equilibrated in buffer A. During the chromatography procedure, in AKTA apparatus, the unadsorbed proteins on the column were rapidly neutralized using the PD-10 equilibrated with 50 mM Tris HCl buffer, pH 7.5 and stored at -80 º C for subsequent assays. Elution of the adsorbed

proteins to the column was done in a 0-100% linear gradient of buffer B (20mM phosphoric acid and NaOH, pH 2.0, 1 M NaCl, 2 mM $CaCl_2$ and 2 mM $MgCl_2$). Gradient formed automatically by mixing buffer A with buffer B. The chromatographic fractionation was carried at a flow rate 1 mL/min of buffer solution and with continuous reading of absorbance at 280 nm. The eluates were collected by visualization of the chromatogram, and the same procedure used in the unbound protein was used here.

For all Mono S column chromatography samples collected were performed hemagglutination assays, as described in 2.2.5.2., in order to detect the presence of lectins. After hemagglutination results analysis, an SDS-PAGE-R gel was performed in order to evaluate the polypeptide profile of the sample that showed hemagglutination activity, which was unadsorbed to the Mono S column fraction.

3.4. Proteomic analysis (2D) of purified lectins binding to HT29 cells membranes

The proteomic analysis of the purified lectins from Mono S column, that after incubation bind to the glycosylated receptors of the HT29 membranes was performed. For evaluate the polypeptide binding, electrophoretic techniques, namely, two-dimensional electrophoresis (2D) was performed. This technique increases the resolution in the separation of proteins in order to permit the identification of its various isoforms, with molecular weight and isoelectric point characterization.

Two-dimensional electrophoresis technique is a compromise of two protein separation techniques, isoelectric focusing (IEF) and a unidimensional electrophoresis (SDS-PAGE-R). The first dimension is the IEF that is based on the amphoteric property and the isoelectric point (pI) of the protein. Proteins are placed on a polyacrylamide gel with a pH gradient (strip) and subjected to an electric field, which migrate until reaching the pH corresponding to their pI. The second dimension is an SDS-PAGE-R, and does not include a concentration gel prior to the separation gel, since proteins are already concentrated in the IEF strip.

The preparation of the sample is essential to free it of salts, detergents, phenols, nucleic acids and polysaccharides, compounds which interfere with the migration of the polypeptides into the gel. For this assay, we test the sample obtained after incubation of the unadsorbed Mono S fraction with HT29 membranes (1 mg of HT29 membranes and 500 µg of unadsorbed Mono S fraction),

and we also used 1 mg of HT29 membranes and 500 µg of unadsorbed Mono S fraction, as control, for membranes and sample, respectively.

All the samples were pre-treated by exchanging extraction buffer for MilliQ water using PD-10 columns (GEHealthcare), and washing (3 to 4 times) by ultrafiltration in centricons (Microsep Advanced Centrifugal Devices, PALL) of cut-off 10 kDa using MilliQ double-distilled water. After three washes the samples were precipitated with acetone and the pellet resuspended in the required volume of IEF solubilisation buffer (7 M urea, 2 M thiourea, 2% (v/v) NP-40, 1% (w/v) DTT), added IPG buffer 4 µL of IPG added to 200 µL of solubilisation buffer, for IEF strips, 11 cm). After solubilisation, the samples were centrifuged at 21,500 *g*, for 10 min (VWR CT15RE). Focusing strips impregnated with immobilins, IPG (immobilized pH gel) strips of 11 cm, forming a pH gradient of 3 to 10, obtained commercially from BioRad, were used.

The protein samples were deposited in the aisles of the IPGphor support (sarcophagus), and the IEF strips were placed on the samples. The gel side was always in contact with the samples and the strips were placed according to the respective poles of the focusing device holder (IPGphor). Thereafter, the carrier (sarcophagus) was placed in the focusing apparatus and rehydration was scheduled overnight for 12 hours, 30 V.h. After the rehydration, the run was performed in six steps programmed as follows: step 1 - 250 V.h, 1 h; Step 2 - 500 V.h., 2 h; Step 3 - 1000 V.h, 2 h; Step 4 - 2500 V.h, 3.5h; Step 5 -8.000 V.h, 1h; Step 6 - 8,000 V.h, 25 min. After the isoelectric focusing was completed, the strip was removed and conditioned in a glass tube and stored at -80 º C until tested for 2D analysis.

After isoelectric focusing (1D-IEF), the strip was incubated in stabilization buffer 50 mM Tris-HCl, pH 8.8, 6 M urea, 2% (w/v) SDS, 30% (v/v) glycerol, 1% (w/v) dithiothreitol (DTT), 15 min at room temperature with gentle shaking. After the first incubation, the strip was re-incubated for DTT removal, for 15 min under mild agitation with a similar solution as above containing 2.5% (w/v) iodoacetamide (replacing dithiothreitol). After the last incubation, the strip was placed on the top of a 17.5% (w/v) acrylamide SDS-PAGE gel, 1 mm thick, sealed with a low electroendosmosis (EEO) agarose solution at 0.5% (w/v), performed in 50 mM Tris-HCl buffer, pH 8.8, containing 0.002% (w/v) bromophenol blue, for frontline visualization during electrophoretic separation. The electrophoretic conditions, initially of 15 mA and 220 V, were established for 15 min for sample entry into the gel, followed by an electric current of 30 mA and a potential difference of 220 V, until the end of the run. After running the gel, it was staining by silver nitrate, as described in 2.2.4..

4. Antitumor activity final evaluation of purified lectins in HT29 cells

4.1. Material

4.1.1. Biological material

Plant material

For this last task, it was used *Arbutus unedo* total protein extract and the fraction of mono S column with hemagglutination activity (unadsorbed Mono S, call from now lectin). These samples were stored in -80 º C freezer in aliquots.

Human material

The same cell line HT29 was used for the screening of the species with antitumor activity.

4.2. Methods

After lectin purification, the antitumor activity was tested. Cell viability and cell death was measure in HT29 cells exposed to 25, 50, 100 and 250 µg/mL of total extract and purified lectin, for 72 h. Cell viability and cell death were evaluated by MTS metabolism and LDH release assays, respectively, as previously described in 1.2.2. of Chapter II.

4.2.1. Morphological evaluation of apoptosis in cultured cells

Apoptotic cells can be identified based on their typical nuclear morphological changes, such as chromatin condensation and nuclear fragmentation. Therefore, nuclear morphology was assessed using the DNA-binding stain Hoechst. This assay provides a rapid and convenient way of assessing apoptosis based on fluorescence detection of nuclear morphological changes characteristic of apoptosis. The blue-fluorescent Hoescht dye 33258 stains chromatin, and normal nuclei are identified as non-condensed chromatin dispersed over the entire nucleus, whereas apoptotic nuclei are characterized by condensed chromatin, contiguous to the nuclear membrane, as well as by nuclear fragmentation of condensed chromatin.

For this purpose, HT29 cells were seeded into 35 mm dishes, at density of 50 x 10^3 cells per dish, and exposed to total protein extract and purified lectin. Attached cells were fixed with 4% (v/v) paraformaldehyde in phosphate-buffer saline (PBS) for 20 min at room temperature, protected

from light. Subsequently, cells were washed with PBS and mounted with coverslips using PBS/glycerol (3:1). Nuclear morphology was evaluated by fluorescence microscopy using an AxioScope.A1 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). Images were acquired, under 400x magnification, using an AxionCam HRm camera with the AxionVision software (release 4.8; both from Carl Zeiss Microscopy GmbH). A minimum of five random microscopic fields with approximately 100 nuclei were counted for each condition, from at least three independent experiments. Fluorescent nuclei were categorized according to the condensation and staining characteristics of chromatin.

4.2.2. Evaluation of caspase-3/7 activity

Caspases are a family of cysteine proteases that play a critical role in programmed cell death, and caspase activation represents one of the earliest measurable events of apoptosis. Accordingly, the detection of effector caspase-3 and -7 activation status is frequently used as a general marker of apoptosis. Caspase-3 and -7 activity was measured using the Caspase-Glo 3/7 Assay (Promega). This assay is based on the cleavage of a proluminescent substrate containing the specific DEVD sequence recognized by caspase-3 and -7 to release aminoluciferin. Generates a luminescent signal directly proportional to the amount of caspases activity present in the sample.

HT29 cells were seeded on a 96-well plate at a 5 x 10^3 cells per well. Total protein extract and purified lectin were incubated, 75 µL of Caspase-Glo 3/7 reagent was added to each well, and plates were mixed by orbital shaking for 30 s. Subsequently, the mixture was incubated at room temperature for 30 min, leading to complete cell lysis, stabilization of substrate cleavage by caspases, and accumulation of luminescent signal detected by the GloMax-Multi+ detection system (Promega).

Statistical analysis

All data were expressed as mean ± Standard Error of Mean (SEM) from at least three independent experiments. Statistical significance was evaluated using the Student's *t* test. Values of $p < 0.05$ were considered significant.

Chapter III – Results and Discussion

1. Screening of plant species with antitumor activity in HT29 cells

The total protein extract from *Juniperus oxycedrus* subsp. *oxycedrus*, *Juniperus oxycedrus* subsp. *badia*, *Arbutus unedo* and *Corema album* were selected based on previous studies that show that all their extracts contain protein-like lectins based on hemagglutination activity. In addition to the hemagglutination activity assay, the wound assay and the inhibition of metalloproteinases MMP2 and MMP9 activities have been tested for these four extracts, also in HT29 cells (Nunes, 2014).

The results showed that *Juniperus oxycedrus* subsp. *badia* was the the most effective at inhibiting cellular invasion, with 33% of invasion of the cut, promoting a cut-off inhibition of approximately 50% compared to the control. This extract also showed a promising inhibition of gelatinase activity as compared to the control, with a reduction of approximately 25% in MMP2 and MMP9 activity being observed. *Arbutus unedo* also showed good results in these two assays, with 26% of invasion of the cut, and with 25% of MMP2 and MMP9 inhibition. For the species *Juniperus oxycedrus* subsp. *oxycedrus* and *Corema album*, only the wound test was performed, showing a cut off percentage of 68% and 41%, respectively, but evidencing a lower percentage inhibition of cell invasion compared to the control (Nunes, 2014).

To continue with the previous work, we investigated cell death in HT29 cells, using the four total protein extracts, *Juniperus oxycedrus* subsp. *oxycedrus* (Joox), *Juniperus oxycedrus* subsp. *badia* (Joba), *Arbutus unedo* (Aun) and *Corema album* (Cal), to then select the most promising species.

Figure III-1 shows that all tested species have the capacity to induce cell death and decrease cell viability. However, there are differences among species. The *Juniperus oxycedrus* subsp. *oxycedrus* protein extract induced a large increase in LDH activity after 48 h, which decreased after 72 h. The MTS metabolism was decreased by about 20 to 25% in relation to control. The *Juniperus oxycedrus* subsp. *badia* protein extract reveals the slightest effect, not checking big difference between the two endpoints in both assays. On the other hand, *Arbutus unedo* and *Corema album* showed very similar results being the samples with increased LDH activity, and this increase was

greater after 72 h. The MTS metabolism assay for these two species showed a good reduction of the MTS metabolism; however, *Corema album* was the species, among the four, with the greatest reduction of MTS metabolism after 72 h.

Since *Arbutus unedo* and *Corema album* total protein extracts here the two species with better results, and in order to select one species, we looked for the previous results obtained in our sample. Therefore, knowing that *Corema album* was less effective in the tumor invasion assay, we selected the *Arbutus unedo* to proceed with lectins purification.

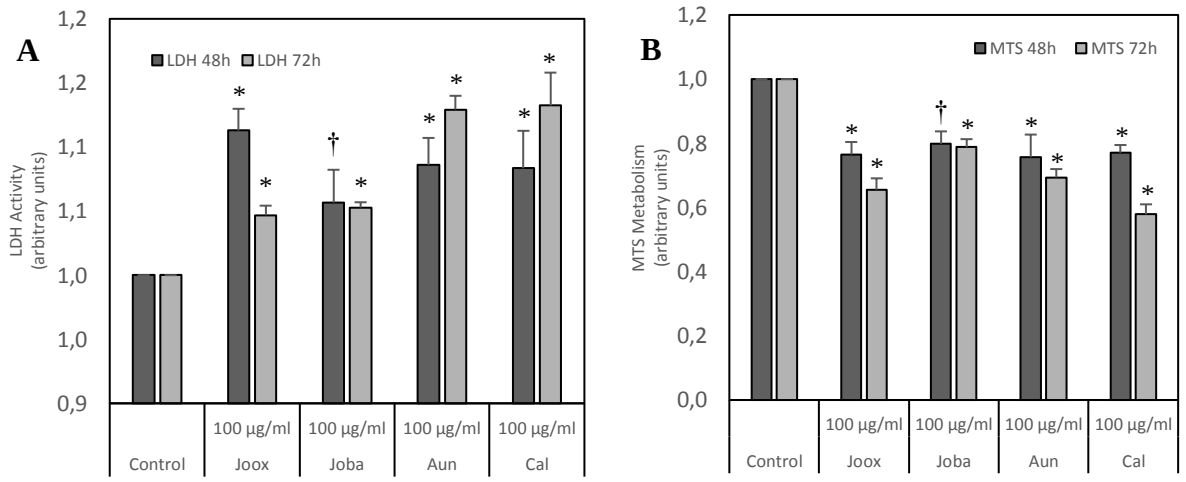


Figure III.1 – Cytotoxicity assays in HT29 cells exposed to 100 µg/mL of total protein extract from *Juniperus oxycedrus* subsp. *oxycedrus* (Joox), *Juniperus oxycedrus* subsp. *badia* (Joba), *Arbutus unedo* (Aun) and *Corema album* (Cal), for 48 and 72 h. Saline containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$ was used as control. General cell death (**A**) and cell viability (**B**) were evaluated by LDH realease and MTS metabolism assays, respectively. Results are expressed as mean ± SEM fold-change to control from three independent experiments *p <0,01 and †<0,05 for HT29 cells.

2. Polypeptide characterization of *Arbutus unedo* leaf extracts

2.1. *Arbutus unedo* total protein extract analysis *vs.* polypeptide binding to HT29 cells

2.1.1. Hemagglutination and inhibition assay

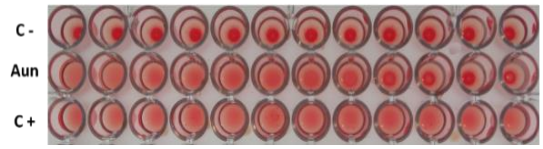
After species selection, we began the assay by confirming the hemagglutination activity and the sugar specificity of the selected total protein extract. For this, we evaluated the hemagglutinating unit of the total extract against rabbit erythrocytes solution at 4% (v/v). Then, using four hemagglutinating units, we were able to see the inhibition of this same activity using a panel of carefully selected sugars.

These sugar selection is related to two main reasons: to try to classify the lectins present in our sample based on the sugar specificity, but also to perceive their specificity to characteristic sugars of the glycomic aberration involved in the tumoral process, namely branched *N*-glycans and fucose.

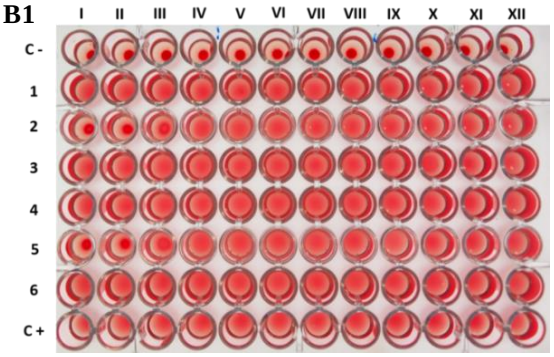
Our results suggest that we have detected three types of lectins in our protein extract, since, according to the sugars inhibition tests, we have inhibition for group IV (*L*-fucose) and the inhibition by glucosamine and galactosamine, could means that core molecules, glucose and galactose, can be part of the others groups like mannose/glucose (group I), galactose-*N*-acetyl-galactosamine (group II) and *N*-acetly-glucosamine (group III), from lectins classification by sugar specificity. This suggests the importance of these proteins in the recognition of tumour cells, since these glycans are importance in tumour development.

In aberrant glycosylation several sugars have a protagonist, as β1,6 branched and bisected *N*-glycan that modulates cell behavior by interfering in the physical and performance properties of glycosylated adhesion molecules on the cell surface; a poly-*N*-acetyllactosamine-containing glycans, that can be potentially recognized by galectins and a outer-chain polyfucosylation and sialyl Lewis^x production that are potentially recognized by the selectins.

A



B1



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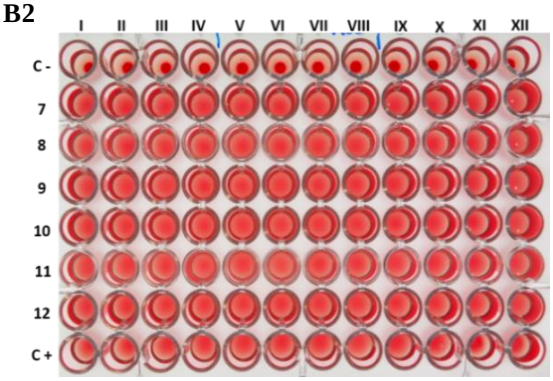
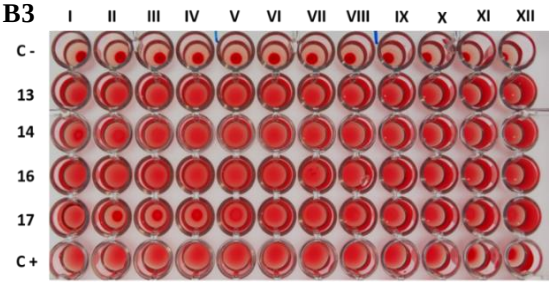


B3



C

Sugar inhibition	Sugar minimal inhibitory concentration (m.i.c.) (mM)	Maximal protein concentration inhibited (4 H.U.) (µg)
1- D-Glucose	-	-
2- D-Glucosamina	11.1	9.16×10^{-2}
3- *N*-Acetyl-D-glucosamine	-	--
4- D-Galactose	-	-
5- D-Galactosamina	11.1	9.16×10^{-2}
6-*N*-Acetly-D-galactosamine	-	-
7- Lactose	-	-
8- D-Mannose	-	-
9- Raffinose	-	-
10- D-Fucose	-	-
11- Melezitose	-	-
12- α-Methly-D-glucopyranoside	-	-
13- α-Metly-D-mannopyranoside	-	-
14- Sucrose	100	9.16×10^{-2}
16- Sialic acid	-	-
17- *L*-Fucose	11.1	9.16×10^{-2}

Figure III.2 – Hemagglutination and inhibition of hemagglutination by sugar assays from *Arbutus unedo* leaves total protein extract. A – Hemagglutination activity: serial dilution (1:3) of total protein extract (50 µg of protein, Aun), negative (C-; saline) and positive (C+; Con A) controls. **B1, B2 and B3** – Hemagglutination activity inhibition by sugar of total protein extract (4 H.U.: 0.0916 µg per well).

Carbohydrates used 1- glucose (0.1 M); 2-glucosamine (0.1 M); 3- *N*-acetyl-D-glucosamine (0.1 M); 4-galactose (0.1 M); 5- galactosamine (0.1 M); 6- *N*-acetyl-D-galactosamine (0.1 M); 7- lactose (0.1 M); 8-mannose (0.1 M); 9- raffinose (0.1 M); 10- D (+) fucose (0.3 M); 11- melezitose (0.1 M); 12- methyl-α-glucopyranoside (0.1 M); methyl-α-mannoside (0.1 M); 14- saccharose (0.1 M); 16- *N*-acetylneuraminic acid (0.1 M) and 17- *L* (-) fucose (0.3 M). **C** – Sugar inhibition of the hemagglutination activity analyses. (-) showed no inhibition of hemagglutination activity.

2.1.2. Polypeptide binding to HT29 cells membranes

Before proceeding with protein purification, we confirmed the proteins extract binding to HT29 cell membranes. For that we incubated protein extract with isolated HT29 membranes and analysed the polypeptide profile of the incubation product by electrophoresis. Results are in Figure III.3.

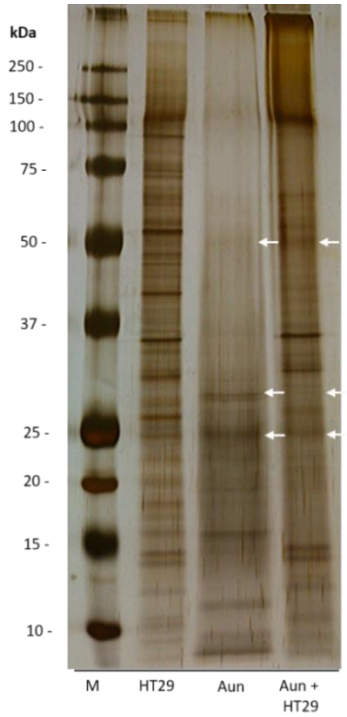


Figure III.3 - Analysis of *Arbutus unedo* proteins extract binding to the HT29 cells membranes. Electrophoretic profile on SDS-PAGE R gel, 17.5% (w/v) acrylamide and $AgNO_3$ staining. Were applied 3 µL of mass molecular marker (M), 13 µg of HT29 isolated membrane (HT29), incubated with saline, 50 µg of *Arbutus unedo* total protein extract (Aun), as membrane and extract controls, respectively, and 13 µg of incubation membrane (Aun + HT29).

The assay of *Arbutus unedo* protein binding to HT29 cell membranes analysis by SDS-PAGE R showed that there were at least 3 bands that bound to HT29 cell membranes with molecules mass of around 25 kDa, 29 kDa and 50 kDa. Since lectins are comprising of 25–30 kDa subunits and as they were in presence of 2-mercaptoethanol, we could be in the presence of 2 subunits of the same lectin or more lectins, if they have not disulphide bonds.

These results can be address only to lectins that bind to glycosylated receptors of HT29 cells membranes, however lectins can be internalized into the cell and act by mitochondrial pathway, what we can´t see by polyacrylamide gel.

3. Arbutus unedo lectins purification by ionic-exchange chromatography

3.1. Anionic-exchange chromatography in Q-Sepharose

After confirming the presence of lectins in the *Arbutus unedo* total protein extract and its binding to HT29 cell membranes, we started the fractionation process using Q-Sepharose (Figure III.4A). The polypeptide profile of all samples collected in this process, unadsorved fraction and eluted polypeptides, were analysed by SDS-PAGE R gel (Figure III.4B). For all samples, the presence of lectin-like proteins was investigated by hemagglutination assays, against a solution of rabbit erythrocytes at 4% (v/v).

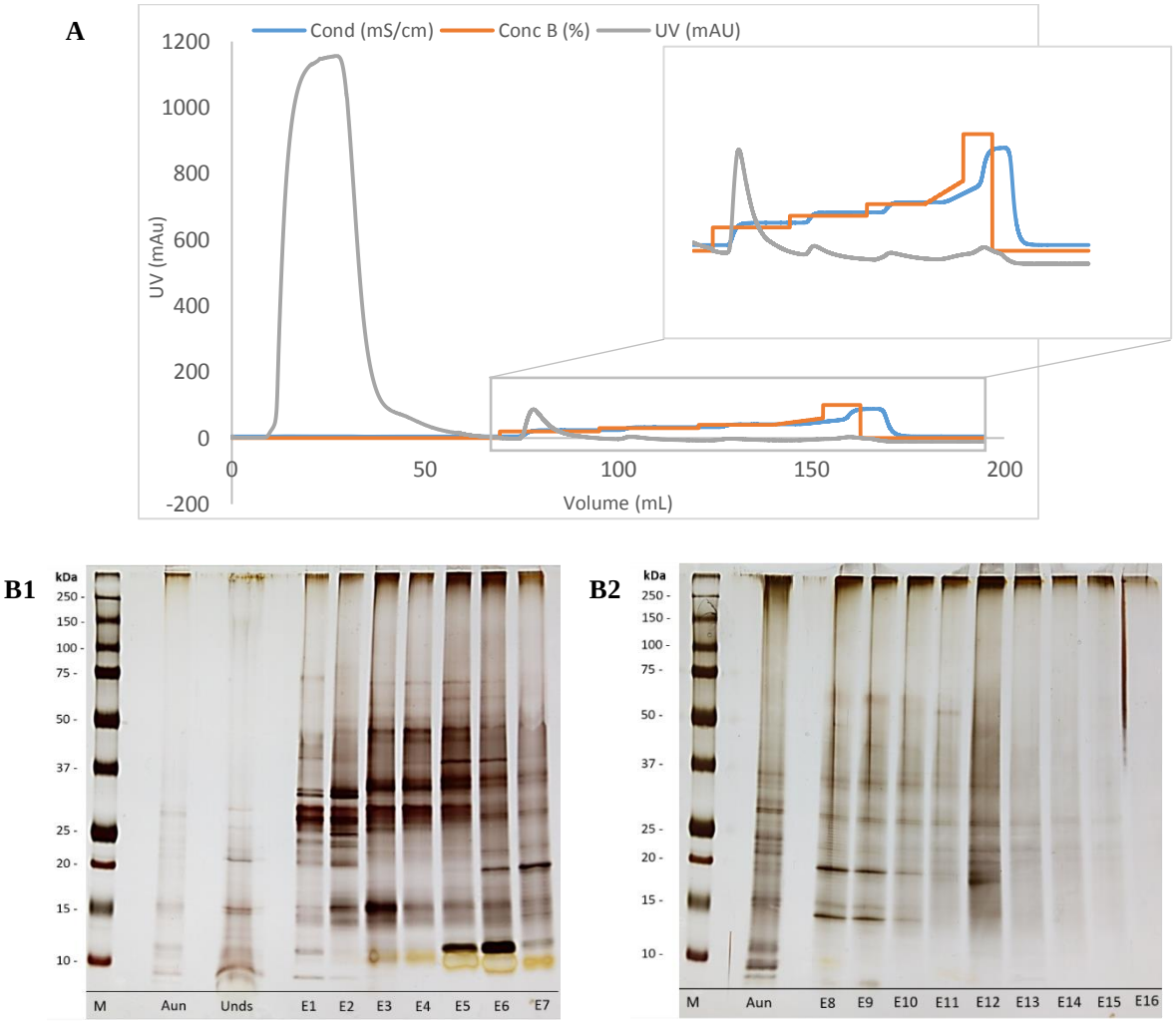


Figure III.4 - *Arbutus unedo* protein fractionation by anionic-exchange chromatography followed SDS-PAGE R gel. A – The total protein extract was applied on a Q-Sepharose column equilibrated in buffer A (50 mM Tris-HCl, pH 7.5, containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$). The buffer B (50 mM Tris-HCl, pH 7.5, 1 M NaCl, containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$) as the elution buffer, with a stepwise gradient: 0-20% step gradient in 4 column volumes (CV), 20-30% in step gradient 4 CV, 30-40% 3 CV, with later linear gradient of 40-60% 2 CV and finally step gradient 60-100% in 3 CV, by adding buffer A and B. The flow of the buffer solution was 1 mL/min. Elution of the proteins was continuously recorded by reading the absorbance at 280 nm. **B1** and **B2** - SDS-PAGE R 17.5% (w/v) acrylamide gels and $AgNO_3$ staining. Polypeptide profile of the diverse eluates collected during fractionation. Were applied 3 µL of molecular mass markers (M), 40 µg of total extract protein of A*rbutus unedo* (Aun); 20 µg of unadsorbed protein fraction and 7 µg of each eluate (E1-E16).

At a first glance the polypeptide profile of some eluates appears promising as some of them have fairly representative bands of the same molecular weight as the bands that bound to the membranes of HT29 cells (Figure III.3). In particular, the eluate 2 shows ≈ 25 and ≈ 29 kDa bands, the eluates 3 to 5 have ≈ 50 kDa band, and in eluates 8 to 12 a band at ≈ 29 kDa is present, however these are not the most representative. In addition to these bands that appear to be the same that than those bind to HT29 cells membrane, another representative bands, namely in eluates 2 and 3, with molecular weights of ≈ 15 kDa, appear to be also compatible with the typical lectin subunit molecular weight.

3.2. Hemagglutination and inhibition of hemagglutination assays

In order to identify the fractions with lectin-like proteins, we carried out the tests with the hemagglutination activity study, applying for each sample 50 μg of protein with 12 dilutions (1:3) and always with a negative control (saline) and a positive control (Con A). This assay was performed against rabbit erythrocytes solution at 4% (v/v). None of the eluates showed hemagglutination activity, however, the unadsorbed Q-Sepharose column fraction showed a very strong hemagglutination activity with a H.U. of 8.47×10^{-4} μg.

We proceeded with the inhibition of hemagglutination activity by sugars study only with unadsorbed Q-Sepharose column fraction (Table III.1). For this sample, the same panel of sugars tested in the *Arbutus unedo* total protein extract was performed in the same conditions.

Table III.1 -Hemagglutination activity and sugars specificity of unadsorbed Q-Sepharose fraction.

Sample	Hemagglutination Unit (H.U.) (µg)	Sugar inhibition (0.1 M)	Sugar minimal inhibitory concentration (m.i.c.) (mM)	Maximal protein concentration inhibited (4 H.U.) (µg)
Unadsorbed Q-Sepharose	8.47×10^{-4}	1- D-Glucose	-	-
		2- D-Glucosamina	3.7	3.39×10^{-3}
		3- *N*-Acetyl-D-glucosamine	-	-
		4- D-Galactose	-	-
		5- D-Galactosamina	1.23	3.39×10^{-3}
		6-*N*-Acetly-D-galactosamine	-	-
		7- Lactose	-	-
		8- D-Mannose	-	-
		9- Raffinose	-	-
		10- D-Fucose*	-	-
		11- Melezitose	-	-
		12- α-Methly-D-glucopyranoside	-	-
		13-α-Metly-D-mannopyranoside	$<0.56 \times 10^{-3}$	3.39×10^{-3}
		14- Sucrose	$<0.56 \times 10^{-3}$	3.39×10^{-3}
		16- Sialic acid	-	-
		17- L-Fucose*	$< 1.7 \times 10^{-3}$	3.39×10^{-3}

*Fucose (0.3 M)

Table III.1 shows the results of hemagglutination activity inhibition by sugars. In addition to sugars that inhibited hemagglutination activity in the total protein extract, α-metly-D-mannopyranoside also showed inhibition the hemagglutination activity for the unadsorbed fraction to the Q-Sepharose column. This shows that the total extract probably has some protein that inhibited the action of lectins with specificity to α-metly-D-mannopyranoside sugar.

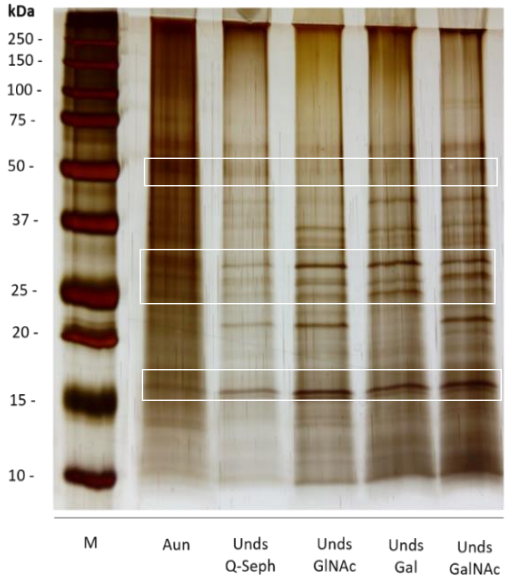
A glycoprotein detection assay for total protein extract, could give a panel of total glycoproteins that can act as inhibitor. These results show that we continue to have lectins from group I, group II and group IV, with one more specific sugar with core mannose, added to core glucose and galactose, according to the classification based on carbohydrate specificity.

3.3. Unadsorbed Q-Sepharose lectins purification by carbohydrate affinity columns

Three affinity columns were used with carbohydrate ligands: *N*-acetyl-D-glucosamine (Vector), D-galactose (Pierce) and *N*-acetyl-D-galactosamine. These columns were selected based on the previous sugars inhibition tests performed on total protein extract and unadsorbed Q-Sepharose fraction.

After carrying out the running on these columns (Figure III.5.A), the hemagglutination activities were performed with rabbit erythrocytes at 4% (v/v) for all eluates (with 50 µg of protein), in order to evaluated the present of lectins (Figure III.5.B). These fractions don't reveal hemagglutination activity from the three affinity columns, however all unadsorbed fractions showed hemagglutination activity. For the samples that had hemagglutination activity the polypeptide profile was evaluated on a SDS-PAGE R gel.

A



B

Fraction (50 µg)	H.U. (µg)
Unadsorbed *N*-Acetyl-D-glucosamine	1,852
Unadsorbed D-Galactose	1,852
Unadsorbed *N*-Acetyl-D-galactosamine	0,2058

Figure III.5 – Polypeptide profile analysis of affinity columns unadsorbed fractions and hemagglutination activity. A - SDS-PAGE R, 17.5% acrylamide (w/v) and $AgNO_3$ staining. Were applied 3 µL of molecular mass markers, 6 µg of *Arbutus unedo* total protein extract (Aun) and 10 µg of unadsorbed fractions, from Q-Sepharose column (Unds Q-Seph), *N*-acetyl-D-glucosamine column (Unds GlNAc), D-

galactose column (Unds Gal) and *N*-acetyl-D-galactosamine column (Unds GalNAc). **B** – Hemagglutination unit (H.U.) from affinity unadsorbed fraction was performed a serial dilution (1:3) of 50 µg of each sample.

The polypeptide profile analysis showed that all unadsorbed fractions have some common bands, in particular the ≈ 29 kDa molecular weight band, who is one of the bands that binds to HT29 cells membranes. However, there is a quite evident band, common to all samples, the ≈ 15 kDa band. The unadsorbed *N*-acetyl-D-galactosamine column sample was the sample with has a stronger band at ≈ 15 kDa and is also the sample with lower hemagglutinating unit (H.U. of 0.2058 µg). These observations may be indicative for a lectin with 15 kDa subunits, since it is commom band to all samples which show hemagglutination activity.

3.4. Unadsorbed Q-Sepharose lectins purification by cationic-exchange chromatography in Mono S column

Since lectins could not be purified by affinity columns, it was decided to proceed with the lectins purification with the unadsorbed Q-Sepharose column fraction. The next purification step was to know what the pH that we can work on Mono S column without loss of sample hemagglutination activity. The sample at different pH (pH 2.0, 2.5, 3.0, 3.5 and 4.0) was tested and it was verified that the low pH did not influence the hemagglutination activity of the sample, the pH 2.0 was selected.

After selecting the working pH, the next step consisted on the application of the unadsorbed Q-Sepharose fraction in the Mono S column (Figure III.6). After collecting the unadsorbed fraction and elution peak fraction the hemagglutination activity was assayed with 50 µg of protein from each fraction (Figure III.7). Only the unadsorbed fraction to Mono S column showed activity, and for that fraction it was performed a SDS-PAGE R gel in order to analysed the polypeptide profile.

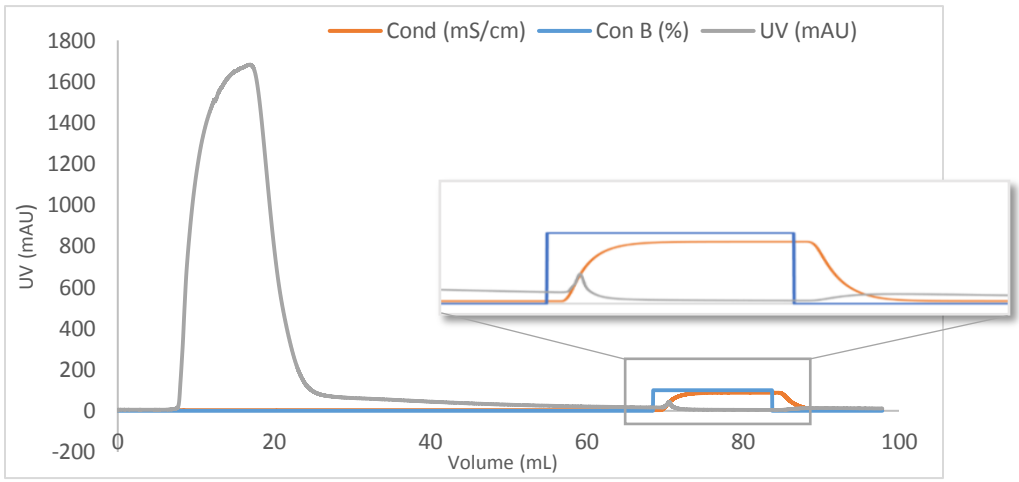


Figure III.6- Representative chromatogram of Mono S column. Proteins fractionation by Mono S column, a strong cation exchanger (Resource S), having a working buffer A (20 mM phosphoric acid and NaOH, pH 2.0, 2 mM $CaCl_2$ and 2 mM $MgCl_2$); with the elution buffer B (20 mM phosphoric acid and NaOH, pH 2.0, 1 M NaCl, 2 mM $CaCl_2$ and 2 mM $MgCl_2$). The flow of the buffer solution was 1 mL/min. Elution of the proteins was continuously recorded by reading the absorbance at 280 nm.

A

Sample (50 µg)	H.U. (µg)
Total protein extract	2.29×10^{-2}
Unadsorbed Q-Sepharose	8.47×10^{-4}
Unadsorbed Mono S	7.6×10^{-3}

B

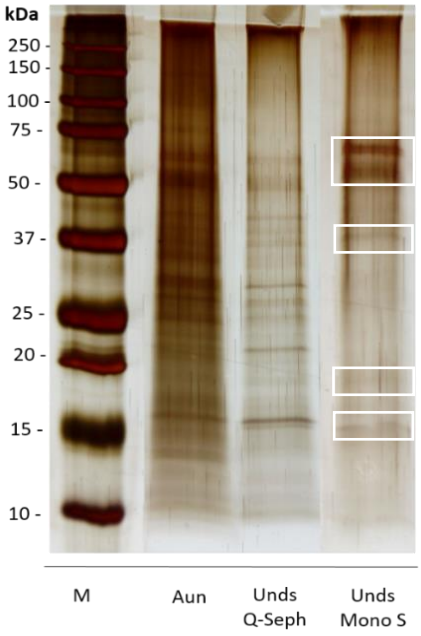


Figure III.7- Unadsorbed Q-Sepharose lectins purification by Mono S column. A - Hemagglutination unit (U.H.) from total protein extract, unadsorbed Q-Sepharose column and unadsorbed Mono S column samples. Serial dilution (1:3) of 50 µg of each sample. **B** - SDS-PAGE R 17.5% (w/v) acrylamide and $AgNO_3$ staining. Were applied 3 µL of molecular mass markers (M), 5 µg of total extract protein of A*rbutus*

unedo (Aun); 11 µg of protein that not bound to the Q-Sepharose column (Unds Q-Seph) and 13 µg of protein that was not bound to the Mono S column (Unds Mono S).

In relation to the hemagglutination activity assay and comparing the three samples present in figure III.7, we can conclude that the unadsorbed fraction to the Q-Sepharose column is the one that presents the best result, in other words, it is the sample that needs the least amount of protein to induce erythrocytes hemagglutination. Perhaps, this fact consists in a lectin that could be denaturated at the work pH of Mono S column, with loss of activity.

The difference between hemagglutination activity of total protein extract and unadsorbed Q-Sepharose fraction, could be addressed to a possible inhibition of lectins activity of extract by proteins like glycoproteins. We can also consider that the preliminary purification on Q-Sepharose column could remove some of these glycoproteins.

When we analyze the polypeptide profile, we find that the Unds Q-Seph sample contains the bands that bound to the HT29 cells membranes, ≈ 25 kDa, ≈ 29 kDa and ≈ 50 kDa, but also has a band at ≈ 15 kDa, with great intensity. This band is the most representative in the unadsorbed Mono S column sample. The ≈ 15 kDa band is also common to unadsorbed affinity column samples, so we can conclude that this band is common to all samples that showed hemagglutination activity, alerting us to the possibility of this band may correspond to a lectin subunit.

4. Proteomic analysis (2D) of purified lectins binding to HT29 cells

Since the profile of the unadsorbed Mono S sample is very simple, and this sample shows good hemagglutination activity, it was decided to carry out the proteomic analysis of the binding of this sample to the HT29 cells membranes glycosylated receptors.

The use of two-dimensional electrophoresis technique in this work aimed to increase the resolution in the separation of proteins in order to allow the identification of its various isoforms, with molecular weight and isoelectric point characterization.

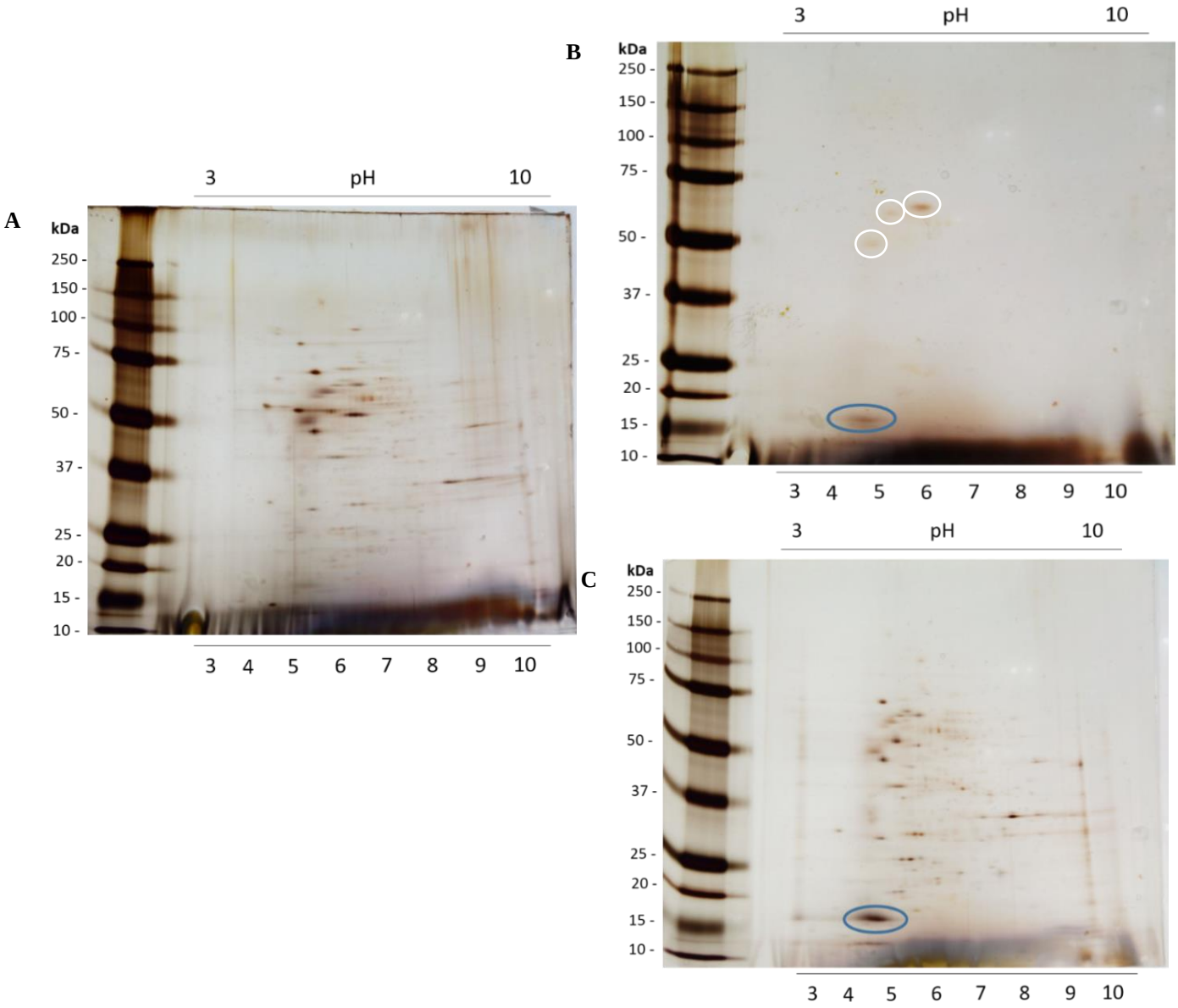


Figure III.8 – Two-dimensional analysis of lectin, present in unadsorbed Mono S fraction, bound to HT29 cell membranes. IEF (1D) with a pH gradient (3-10 pH) performed for all samples. For HT29 membranes 1 mg of protein was applied (**A**), for unadsorbed Mono S fraction it was applied 500 µg of protein (**B**) and 1500 µg of total protein for unadsorbed fraction (500 µg) incubated with HT29 cells membrane (1 mg) (**C**). The strips were overlapped onto an SDS-PAGE with 17.5% (w/v) acrylamide; the gel was staining by $AgNO_3$.

The results of the unadsorbed Mono S proteins that bind to the HT29 cell membranes and the in resolution by two-dimensional analysis show that the proteomic profile of the purified sample is in fact very simple, presenting predominant four peptide spots. The most representative peptide has a molecular weight of ≈ 15 kDa, with pI between 4.5 and 5, then there are two spots with molecular masses between ≈ 60-63 kDa and pI between 5 and 6 and finally, and with less representativeness, a spot with molecular mass of ≈ 50 kDa and pI between 4.5 and 5. All polypeptide spots are acidic.

When we compared the polypeptide gel profile in which we applied the binding sample with the control gel (Figure III.8.A), which represents the proteins membranes profile, we noticed that there is a band (Figure III.8.C) which is not part of the membranes profile, but belongs to the purified sample profile (Figure III.8.B), the band of molecular weight of ≈ 15 kDa with pI between 4.5 and 5. In fact, when we look at the polypeptide profile of the other studied samples that had hemagglutination activity, we see that this band, with molecular weight of ≈ 15 kDa, is always present.

5. Final evaluation of antitumor activity of purified lectin(s) in HT29 cells

The purified lectins (unadsorbed proteins to Mono S column) from *Arbutus unedo* leaves protein extract were assayed for antitumoral activity. For this purpose, HT29 cells were exposed to 25, 50, 100 and 250 µg/mL of total protein extract and to purified lectin, for 72 h. Cell viability and cell death were evaluated by MTS metabolism and LDH release assays, respectively (Figure III.9).

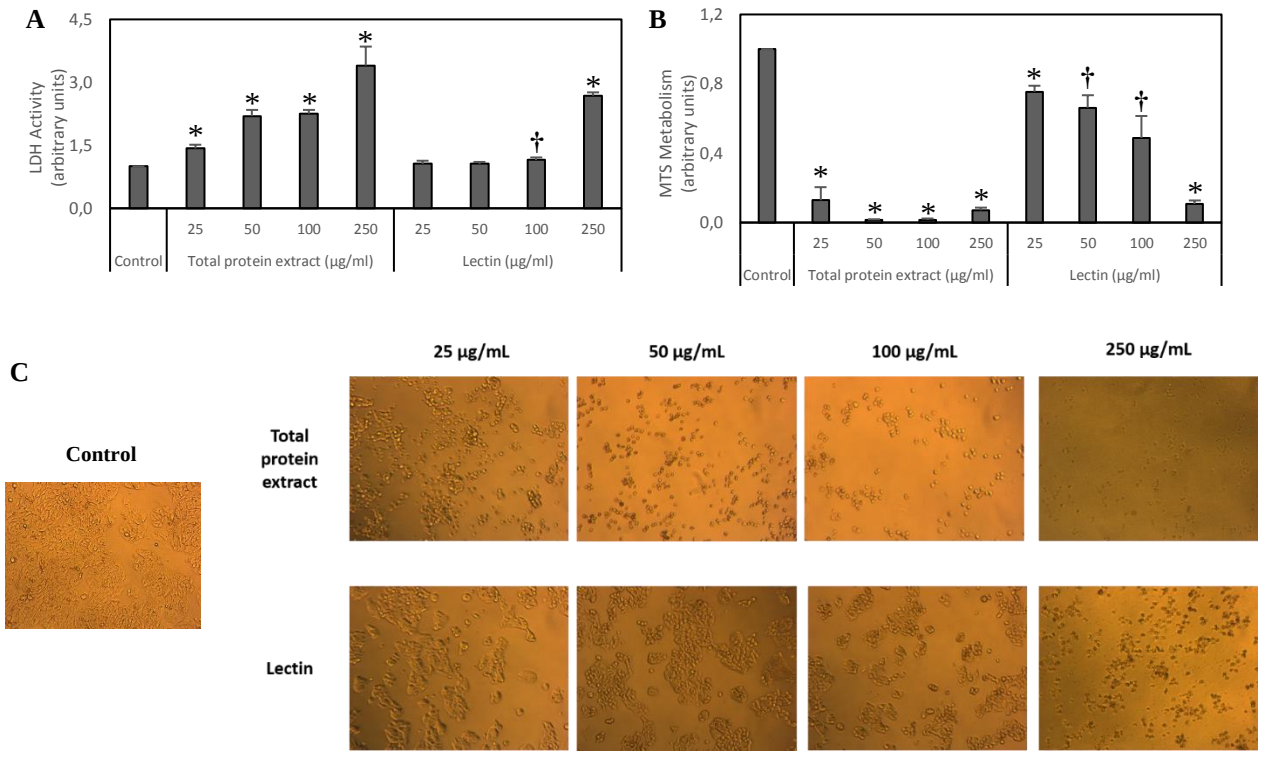


Figure III.9 – Cytotoxicity evaluation of total protein extract and purified lectins in HT29 cells. HT29 were exposed to 25, 50, 100 and 250 µg/mL of total extract and purified lectin or no addition (control) for 72 h. Saline containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$ was used as a control. General cell death **(A)** and cell viability (**B**) were evaluated by LDH release and MTS metabolism assays, respectively. Representative microscopy images at 100x original magnification (**C**). Results are expressed as mean ± SEM fold-change to control from three independent experiments *p <0.01 and †<0.05 from control HT29 cells.

Our results show that total protein extract and purified lectins have the ability to induce cell death and decrease MTS metabolism in a concentration dependent manner (Figure III.9.A e B). However, the total protein extract induces more general cell death, and a strong decrease in MTS metabolism. In addition, Figure III.9.C shows that the total protein extract at 100 µg/mL drastically reduces the number of cells. At 250 µg/mL, the few cells still present are extremely picnotic.

When we analyzed the results for the purified sample we found that both the increase in LDH activity and the decrease in the MTS metabolism are less pronounced than those of the total protein extract (Figure III.9.A e B). We may conclude that the total protein extract may have other proteins, in addition to lectin, which may also be of lectin type (synergism) that strongly increased cytotoxicity.

It is well established that lectins are potent proteins with antitumoral activity due to apoptosis (Yau *et al.*, 2015). Activation of caspases has been shown to be a hallmark of lectin-induced cancer cell by apoptosis (Shi *et al.*, 2016). Therefore, caspase-3/7 activation was evaluated, using HT29 cells exposed to either 25 µg/mL of total protein extract, 25 or 100 µg/mL of purified lectin, or no addition (control) for 24 h (Figure III.10.A). Moreover, changes in nuclear morphology were evaluated by fluorescence microscopy of Hoechst stained nuclei, following exposure to either 25 µg/mL of total protein extract, 100 µg/mL of purified lectin, or no addition (control) for 24 h, to detect apoptotic nuclei (Figure III.10.B).

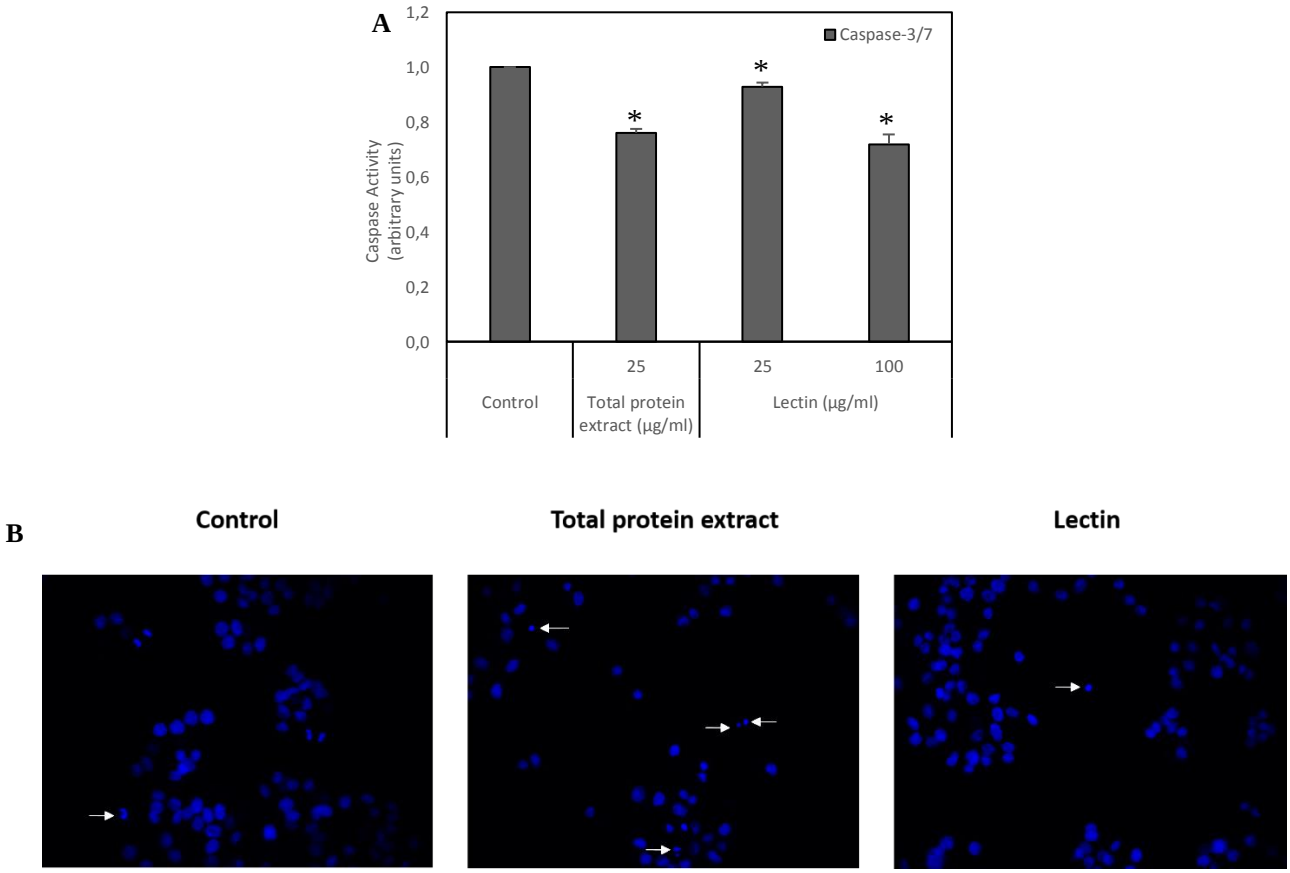


Figure III.10 – Assessment of apoptosis induced by total protein extract and purified lectins in HT29 cells. Cells were exposed to 25 µg/mL of total protein extract, 100 µg/mL of purified lectin or no addition (control) for 72 h. Saline containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$ was used as a control. (**A)** Caspase-3/7 activity was determined at 24 h after cell exposure. (**B**) Nuclear morphology was evaluated after Hoechst

staining by fluorescence microscopy at 24 h of cell exposure. Representative images of Hoechst staining at 400x original magnification. Arrows indicate nuclear fragmentation and chromatin condensation. Results expressed as mean ± SEM fold-change to control, from 3 independent experiments. *p <0.01 from HT29 cells.

The results suggest that under these experimental conditions, caspases are not activated and nuclear fragmentation is also not evident. Nevertheless, more studies are needed to conclude that apoptosis in not involved in lectins cytotoxicity.

Chapter IV – Final conclusions and future perspectives

The work developed in this thesis allowed to purify lectin(s) from leaves of *Arbutus unedo* total protein extract that exhibit antitumor activity and lectin activity. It was concluded that purified lectin(s) has the ability to induce cell death and decrease MTS metabolism.

Based on our results we can conclude that the purified lectin, with ≈ 15 kDa molecular weight, binds to glycosylated receptor in the HT29 cells surface and most likely induces cell death. We may also suggest that the *Arbutus unedo* total protein extract has another(s) lectin(s) that can act like antitumoral agents but by inhibition of cellular proliferation, based on the results of MMP2 and MMP9 inhibition assays, determined in previous work, associated with the LDH and MTS results of total protein extract. These results reveal that this species is an excellent reservoir of lectins with antitumor activity once *Arbutus unedo* leaves contain lectins that are antitumoral agents. They may act by synergic mechanisms with inhibition of cellular proliferation that can be used in the metastatic process control of the and cellular death by necrosis.

The purified lectin with a molecular weight of ≈ 15 kDa, should be sequenced. Since it was not possible to determine the inhibition of hemagglutination activity by sugars, it would be interesting to do so. After this determination, it could still be ascertained whether this lectin has the ability to induce death in normal colon fibroblasts, to determine the binding specificity of this protein. Although cell death may not occur through apoptosis, this protein may be interesting as an identifier of tumor cells, or function as a drug delivery tool, in case specificity for the glycosylated receptors of tumor cells is demonstrated. Binding to these cells would be specific and could be detected at the beginning of the disease.

Throughout the separation process, other lectins, capable of binding to the HT29 cells membranes, have been detected. Therefore, this species may contain other lectins, namely, lectins that did not resist to pH 2. It would be interesting to have another purification method that does not involve the use of low pH solutions. After further purification, we would repeat all hemagglutination activity assays and the inhibition by sugars for the new fractions.

In summary, lectins are proteins with excellent antitumor capacity. There are still many species in our flora to study further, which may be promising in relation to the presence of lectins with these capacities.

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